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Analytical Approaches to Unravel the Endogenous Formation and Biotransformation of Prednisolone in Livestock

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NOTATION INDEX

Δ^4-ADD	1,4-androstiene-3,17-dione
Δ^1-SDH	Δ^1-steroid-dehydrogenase
ACTH	Adrenocorticotrophic hormone
AGC	Automatic gain control
AIF	All ion fragmentation
ANOVA	One-way analysis of variance
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
AUC	Area under curve
CC_α	Decision limits
CC_β	Detection capabilities
CCF	Central composite face
CD	Commission decision
CDS	Calibrant delivery system
CER	Centre d'Economie Rurale
CFU	Colony-forming unit
CRH	Corticotropin-releasing hormone
CV	Cross-validated
DDA	Data-dependent acquisition
DHEA	dehydroepiandrosteron
DHP	dihydroprednisolone
DIA	Data-independent acquisition
EC	European Commission
EIA	Enzyme immunoassays
ESI	Electrospray ionization
EU	European Union
EURL	European reference laboratories
FT-ICR	Fourier transform ion cyclotron resonance
FWHM	Full width at half maximum
GC	Gas Chromatography
HCA	Hierarchical cluster analysis
HCD	High energy collisional dissociation
HESI	Heated electrospray ionization

HLB	Hydrophilic-lipophilic balance
HPA	Hypothalamus-pituitary-adrenocortical
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
HSD	Hydroxysteroid dehydrogenase
IDA	Information-dependent acquisition
ILVO	Institute for Agricultural and Fisheries Research
IRMS	Isotope ratio mass spectrometry
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LTQ	Linear ion trap quadrupole
m/z	Mass-to-charge ratio
MAX	Mixed-mode, reversed-phase/strong anion-exchange
mmu	Millimass units
MRL	Maximum residue limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSX-DIA	Multiplexed MS/MS data-independent acquisition
NMR	Nuclear magnetic resonance
No	Number
NH_3	Ammonia
OPLS-DA	Othogonal partial least squares discriminant analysis
ppm	Parts per million
PCA	Principal component analysis
QqQ-MS/MS	Quadrupole tandem mass spectrometry
QuEChERS	Quick, easy, cheap, effective, rugged and safe
RIA	Radioimmunoassay
ROC	Receiver-operating characteristic
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SRM	Selected reaction-monitoring
SUS	Shared and unique structure
SWATH	Sequential window acquisition of all theoretical mass spectra

TBME	Tert-butyl methylether
ToF	Time of flight
UHPLC	Ultra high-performance liquid chromatography
VIP	Variable importance in projection
WO	Washout

CHAPTER I:

GENERAL INTRODUCTION

1. Glucocorticoids

Cortisol and cortisone are steroid hormones naturally produced in the adrenal cortex. Since their discovery in the 1940s, the recognition of their anti-inflammatory properties has led to the development of synthetic glucocorticoid analogues, which exert even higher anti-inflammatory activities i.e. betamethasone, dexamethasone, methylprednisolone and prednisolone, with prednisone as prodrug [1]. Besides their anti-inflammatory properties, these drugs also induce body weight gain in production animals. Since the early 1950s they became very popular as growth-promoting agents in livestock [2].

1.1. Origin and chemical configuration

1.1.1. Natural glucocorticoids

Cortisol is the main natural glucocorticoid and its circulating level is maintained by the hypothalamus-pituitary-adrenocortical (HPA) axis (Figure 1.1.A). Small-bodied neurons in the paraventricular nucleus of the hypothalamus secrete the neuropeptide corticotropin-releasing hormone (CRH) that leads to the release of preformed adrenocorticotropic hormone (ACTH or corticotropine) in the neighbouring anterior pituitary gland. ACTH enhances the conversion of cholesterol to cortisol, mainly by cytochrome P450 enzymes in the adrenal gland. The series of hydroxylation steps required to form cortisol take place in the *zona fasciculata* of the adrenal cortex, which defines the outer layer of the gland (Figure 1.1.B). Afterwards, cortisol diffuses out of the cells into the plasma where most of the circulating cortisol is bound to proteins. Approximately 90% is transported by the corticosteroid-binding globulin, also known as transcortin. An additional 7% is bound to albumin and only 3% to 4% of the circulating cortisol is free. Only the unbound fraction can pass through the cell membranes and bind to intracellular cortisol receptors while mediating corticosteroid effects [3].

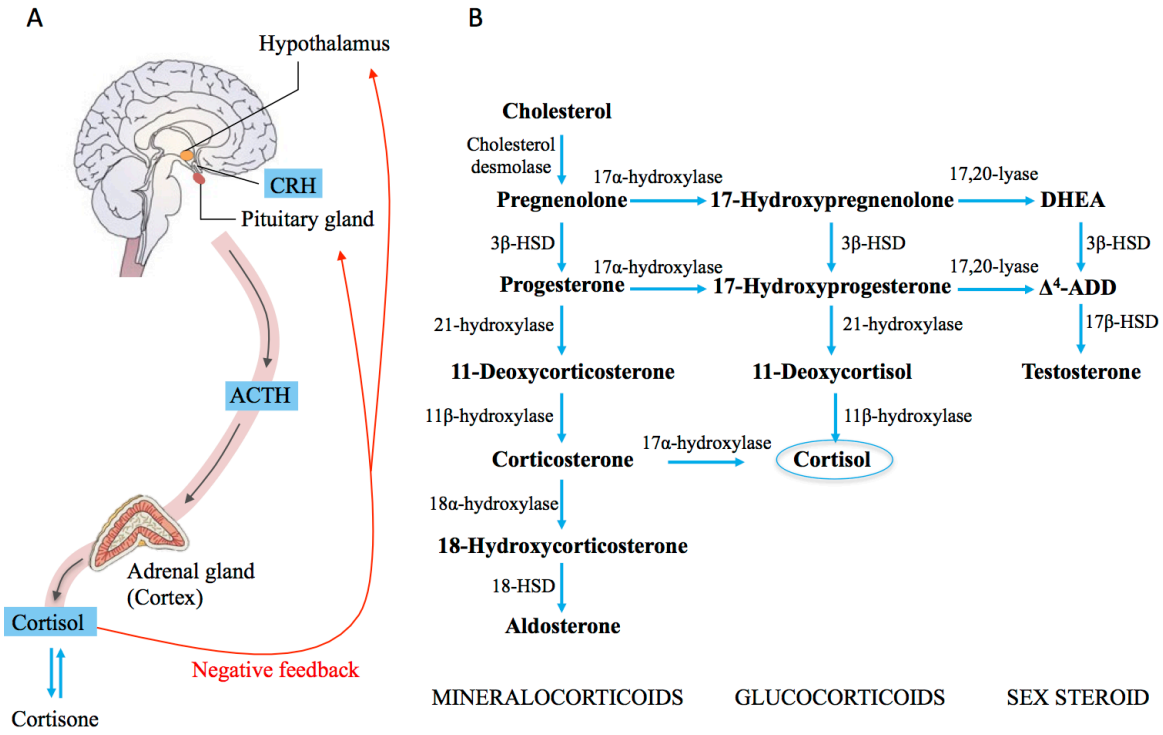

Figure 1.1. The hypothalamic-pituitary-adrenocortical axis and its negative feedback circuit enables to provide a continuous and equilibrated release of glucocorticoids (A) (after Eisenberger and Cole, 2012) [4]. Schematic summary of the biosynthesis of the adrenal steroids, the mineralocorticoid aldosterone, the glucocorticoid cortisol and the sex hormone testosterone from cholesterol with the help of cholesterol desmolase (CYP11A1), 17α-hydroxylase (CYP17A1), 17,20-lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase, 21-hydroxylase (CYP21A2), 11β-hydroxylase (CYP11B1), 18-hydroxylase (CYP11B2), 17β-hydroxysteroid dehydrogenase (B) (after Ortsäter *et al.*, 2012) [5]. CRH: corticotropin-releasing hormone; ACTH: adrenocorticotropic hormone; DHEA: dehydroepiandrosteron; Δ^4-ADD: 1,4-androstadiene-3,17-dione.

To maintain the circulating glucocorticoid levels, CRH is secreted with a frequency of two or three secretory episodes per hour in a circadian, pulsatile manner, with greater pulse amplitudes in the early morning. This can be modulated by changes in light intensities, feeding schedules, activity and stress [6][7]. Secreted cortisol has a negative feedback at multiple levels of the HPA axis. Stress such as injury, infection, cold, pain and fear can override the normal negative feedback control mechanisms, leading to increased plasma concentrations of cortisol [8][9].

Cortisol is partially converted into cortisone by the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD) enzyme. The glucocorticoid activity of this metabolite is however much lower, hence one sometimes refers to it as the hormonal inactive metabolite of cortisol [5]. In time of stress, cortisone can be rapidly reconverted to cortisol by the action of the 11β-HSD type 1, which acts

predominantly as an 11-oxo-reductase [10]. Unlike cortisol, cortisone only occurs in unbound form in the plasma [11].

The natural glucocorticoid, cortisol, has a double bond in the 4,5 position and a 3-keto group on ring A, an 11β-hydroxyl group on ring C, 17α-hydroxyl group on ring D and a 20-keto group (Figure 1.2.).

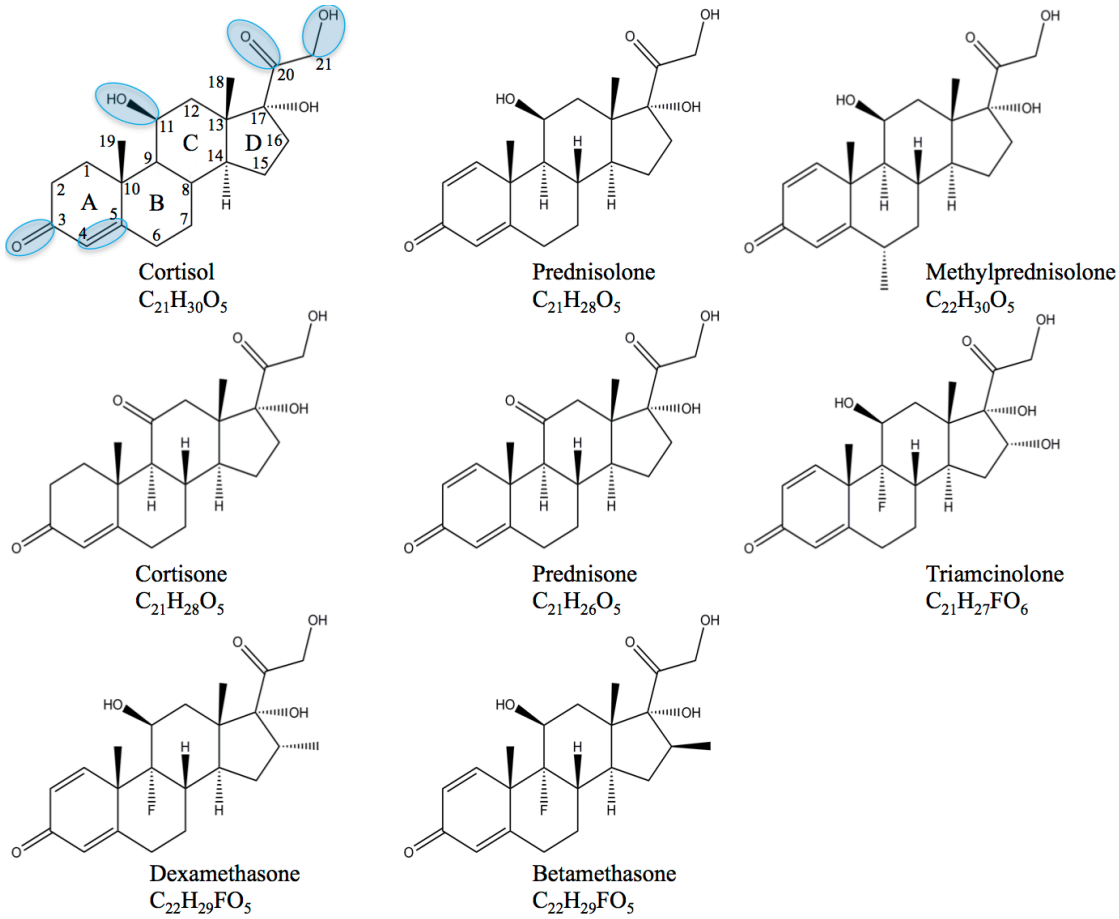

Figure 1.2. The important structural sites, which determine the activity of a glucocorticoid base, are shown on the structure of cortisol, together with structures of common glucocorticoids used in veterinary medicine (after Brunton *et al.*, 2006) [3].

1.1.2. Synthetic glucocorticoids

Chemical modifications of the cortisol molecule have generated a range of synthetic analogues, which react more specifically and exert a more potent glucocorticoid activity with different pharmacokinetic and pharmacodynamic properties. For example, introduction of an additional double bond in the 1,2-position of ring A, results in the formation of prednisolone or prednisone (Figure 1.2). This double bond in combination with 9α-fluoro derivatives results in the formation of dexamethasone, betamethasone and triamcinolone. Besides, other substitutions such as a

6α-methyl group, may as well influence the glucocorticoid receptor sensitivity. Steroids with an 11-keto substituent, i.e. cortisone and prednisone, must be enzymatically reduced in the liver by 11β-HSD type 1 to the corresponding 11β-hydroxyl group, before they are biologically active [3]. Hydrocortisone is chemically identical to cortisol, but is used to distinguish drug administration from endogenous production [12].

1.2. Metabolism and excretion

Glucocorticoids are inactivated and removed from the circulation by the liver or intestine in a two-step process where a sequential addition of oxygen or hydrogen atoms (phase I metabolism) is followed by the conjugation to more polar glucuronides and sulphates (phase II metabolism) in order to be excreted via urine or faeces [13].

1.2.1. Phase I metabolism

Endogenous cortisol has a short half-life and is converted into cortisone or is inactivated by reduction of the 4,5 double bond in the A-ring. This can occur hepatically or extrahepatically (i.e. kidney). Reduction of the 3-ketone substituent to the 3-hydroxyl derivative only occurs in the liver (Figure 1.3.) [14].

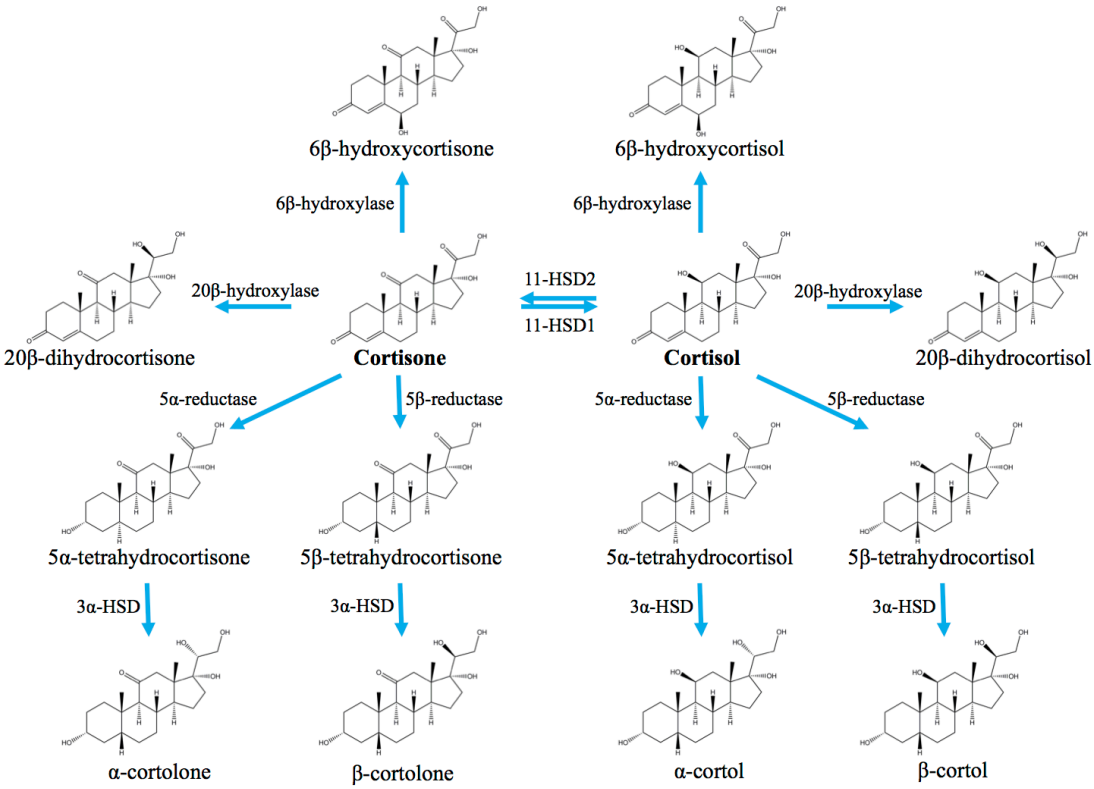

Figure 1.3. The principal biotransformation of cortisol and cortisone performed by cytochrome P450 isoenzymes in bovines (after Remer *et al.*, 2006) [17].

In addition, a side-chain cleavage was found for cortisol, which resulted in the formation of 11,17-dioxoandrostane, which is a typical faecal glucocorticoid metabolite [15]. Cortisone can be metabolized in an identical fashion to cortisol. Both cortisol and cortisone are excreted in urine, whereby a certain fraction retains its initial structural configuration [16].

Synthetic glucocorticoids can also be partially biotransformed before excretion. The biotransformation of prednisolone to 20α- and 20β-dihydroprednisolone, and 6β-hydroxyprednisolone relies more on the oxidative metabolism than the reductive one [18][19]. The urinary excretion profiles of methylprednisolone treated animals, revealed 15 phase I metabolites [20]. Several metabolites were also determined for dexamethasone in urine (e.g. 17-oxodexamethasone, 20-dihydrodexamethasone, 6-hydroxydexamethasone) [21].

1.2.2. Phase II metabolism

In humans, most of these A-ring-reduced steroids are conjugated through the 3-hydroxyl group with a sulphate or glucuronide through enzymatic reactions that take place in the liver, and to a lesser extent in the kidney. The resultant glucuronides (95%) and sulphate esters (4%) are water-soluble and are the predominant excreted metabolites in urine. Only 1% was reported as unconjugated [22]. Neither biliary nor faecal excretion is of quantitative importance in humans [3]. This is in strong contrast with cattle, where the relative proportion of unconjugated glucocorticoids in urine are the most abundant (Table 1.1.).

Table 1.1. Relative proportions (%) of free, glucuronide and sulphate forms for cortisol, tetrahydrocortisol and dexamethasone in bovine urine [23].

	Cortisol	Tetrahydrocortisol	Dexamethasone
Unconjugated	91 - 98%	35 - 59%	72 - 96%
Glucuronide	0.0 - 0.4%	37 - 61%	1.0 - 10%
Sulphate	1.0 - 8.0%	2.0 - 3.0%	3.0 - 17%

1.2.3. Urinary and faecal excretion

Plasma is filtered in the glomerular endothelium of the kidney, where water and electrolytes are passively and actively reabsorbed into the circulation. However, polar compounds, including conjugated glucocorticoids, cannot diffuse back into the circulation and are excreted via urine. This is the predominating excretory route for glucocorticoids, but high interspecies differences

were found. In sheep, 72% of the glucocorticoids are excreted in urine, while in horses and pigs 59% and 93%, respectively [24].

Besides, conjugated glucocorticoids i.e. glucuronides are excreted via the bile into the gut. Once in the intestine, microbial flora under anaerobic conditions, can deconjugate a fraction of the glucocorticoids, which makes reabsorption in the circulation possible [25]. This is the so called enterohepatic circulation. The part that stays in the gut is excreted in the faeces. In farm animals, cortisol excretion via faeces is 28% in sheep, 41% in horses and only 7% in pigs [26].

1.3. Function in the body

Glucocorticoids exert a dual role in the body, which is translated into metabolic and immunological responses. As such glucocorticoids participate in the control of whole body homeostasis and the organism's response to stress.

Glucocorticoids are responsible for modulating the carbohydrate metabolism via mobilization of glucose towards the systemic circulation by stimulating the action of other hyperglycemic hormones for glycogen breakdown (e.g. glucagon, catecholamines and growth hormone). This results in the release of glucose from the hepatocytes. Besides, glucocorticoids inhibit the uptake and utilization of glucose in skeletal muscle and adipose tissue by interfering with insulin signalling [5]. Additionally, glucocorticoids induce gluconeogenesis by promoting muscle atrophy via reduction of protein synthesis and degradation of protein into amino acids. An increased rate of protein metabolism leads to an increased urinary nitrogen excretion and the induction of urea cycle enzymes [27]. Glucocorticoids also enhance gluconeogenesis, through the increased breakdown of triglycerides in adipose tissues, which provide energy and substrates for gluconeogenesis (Figure 1.4.) [5].

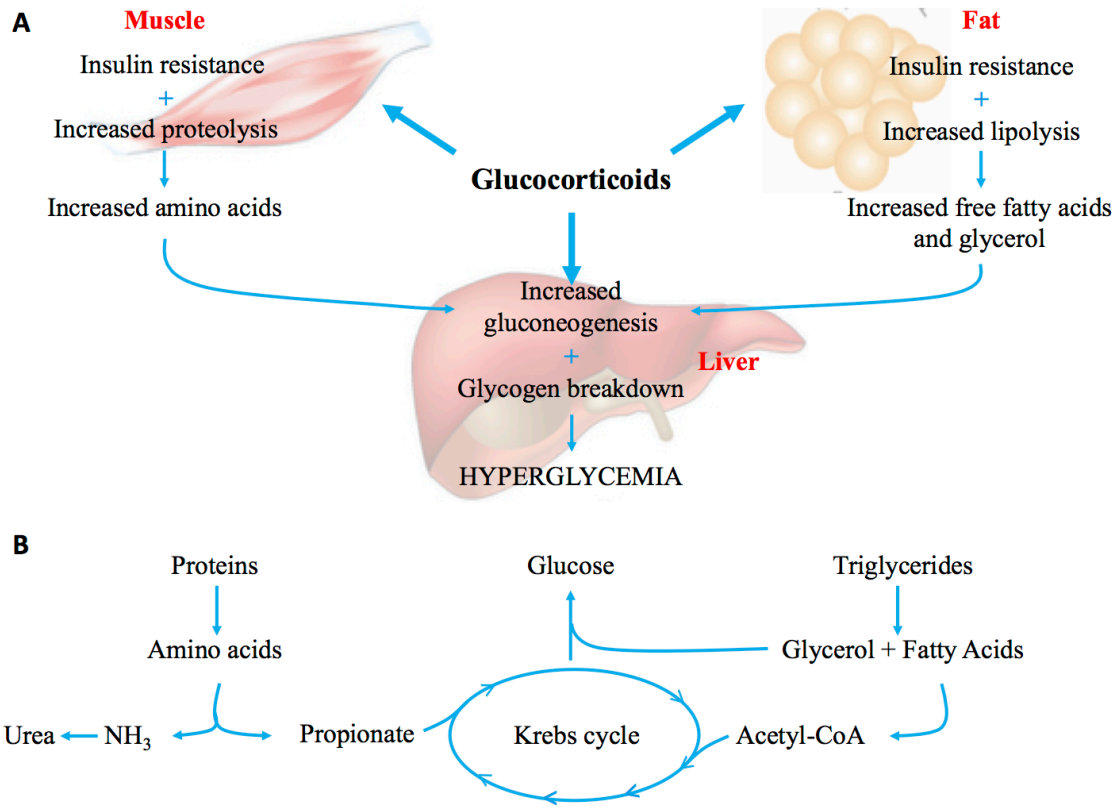

Figure 1.4. Influence of glucocorticoids on carbohydrate metabolism (A), in detail (B).

In addition to their metabolic effects, glucocorticoids also have strong immunosuppressive, anti-inflammatory and anti-allergic properties. In response to injury or infection, the inflammatory response is a primary defence mechanism to restore homeostasis. Inflammation is initiated at the site of injury by resident mast cells and macrophages, which release pro-inflammatory mediators including cytokines (e.g. tumour necrosis factor-alpha, Interleukin-1). These cause the typical inflammatory reaction including vasodilation, increased capillary permeability (humoral response) and leukocyte emigration into injured tissues (cellular response). Different cell types are rapidly attracted to the inflamed site e.g. granulocytes. Antigen presenting cells will bind foreign antigens and present them in the lymph nodes where they instruct the adaptive immune response. Glucocorticoids inhibit many of these initial events such as the inhibition of the vasodilation and the increased vascular permeability. In addition, they decrease leukocyte emigration into inflamed sites [28]. The anti-inflammatory and immunosuppressive actions of glucocorticoids are exerted through down-regulation of the expression of pro-inflammatory cytokines, chemokines, etc. [29][30]. Additionally, apoptosis of macrophages, dendritic cells and T cells, is promoted, which leads to an inhibition of immune responses [31].

Although the use of glucocorticoids does not address the underlying cause of the disease, the major pharmacological use of glucocorticoids is their anti-inflammatory and immunosuppressive action. Depending on the chemical modification, derivatives with more selective glucocorticoid activity, greater potencies and longer pharmacological activity were synthesized (Table 1.2.) [32]. Prednisolone is widely used, particularly for its effectiveness in the treatment of mastitis, this in combination with antimicrobial drugs [33]. Dexamethasone is much more potent and presents even longer pharmacological activity than prednisolone and 6α-methylprednisolone. Given its high potency, long-term treatment with dexamethasone is associated with severe HPA axis suppression. Therefore, it is generally reserved for short-term use in acute conditions [34].

Table 1.2. Relative potencies of natural and synthetic glucocorticoids [32].

Compound	**Anti-inflammatory potency**	**Duration of effect (hours)**	**HPA axis suppression** [a]
Short-acting			
Cortisol	1	8-12	+
Cortisone	0.8	8-12	+
Intermediate-acting			
Prednisone	4	12-36	+
Prednisolone	4	12-36	+
6α-Methylprednisolone	5	12-36	+
Triamcinolone	5	12-36	++
Long-acting			
Betamethasone	25	36-72	+++
Dexamethasone	25	36-72	+++

[a] HPA axis suppression equals the anti-inflammatory potency in most cases.

Besides their broad therapeutic use, glucocorticoids induce side effects on several tissues and organs depending on the dose and duration of use (Figure 1.5.). Moreover, glucocorticoids inhibit the pituitary growth hormone, gonadotropin and thyrotropin secretion and make the target tissues of sex steroids and growth factors resistant to these hormones [3]. A hormonal disorder caused by prolonged exposure of body tissues to high levels of cortisol is the Cushing's syndrome. This disease leads to the symptoms presented in figure 1.5. [35].

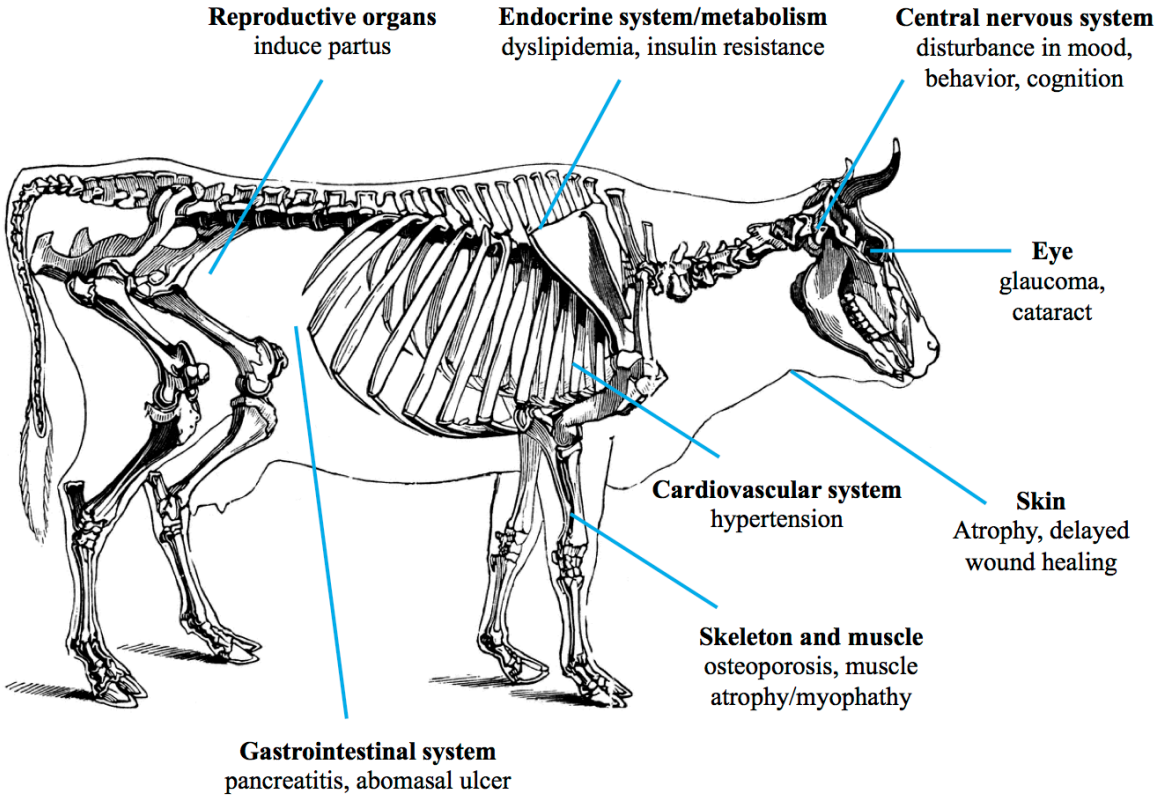

Figure 1.5. Some typical side effects resulting from long-term glucocorticoid treatment in cattle (after Barth, 2006; Lui *et al.*, 2013) [34][36].

Although large doses of synthetic glucocorticoids reduce growth rates and lead to muscle atrophy, they have found a place among growth-promoting additives in meat producing animals. Originally glucocorticoids were often combined with β-agonists and/or anabolic steroids in order to prevent receptor down-regulation and tolerance in the animal or to affect meat quality by increasing water content [37]. However, recently low dosage administration of glucocorticoids has gained popularity. This has no therapeutic purpose but results in improved feed intake, increased live weight gain, reduced feed conversion ratio, reduced nitrogen retention, increased water retention and fat content [37][38][39]. As for dexamethasone (0.4 - 0.7 mg/day) and prednisolone (15 - 30 mg/day), growth-promoting effects have been demonstrated after oral administration during more than 30 days [40]. After illicit treatment, appropriate withdrawal periods are rarely applied, which means that potential harmful residues may be present in animal derived food products [33].

1.4. Legislation

In order to protect consumers against exposure to specific substances present in animal derived food products, the therapeutic use of synthetic glucocorticoids in livestock has been strictly regulated in the European Union. Certain withdrawal periods have to be respected between treatment and slaughtering. To this purpose, the EU has introduced Maximum Residue Limits (MRLs) for betamethasone, dexamethasone, methylprednisolone and prednisolone in various edible tissues of animal origin [41] (Table 1.3.). This is the maximum concentration of residue, resulting from therapeutic use, which is legally permitted in animal derived food products. Obviously, the use of synthetic glucocorticoids for the sole purpose of increasing the body weight of bovines is prohibited in the European Union.

Table 1.3. Pharmacologically active substances and their maximum residue limits (MRLs) [41][42].

Compound	Animal species	MRLs	Target tissues
Betamethasone	Bovine	0.75 μg kg^{-1}	Muscle
		2.0 μg kg^{-1}	Liver
		0.75 μg kg^{-1}	Kidney
		0.3 μg kg^{-1}	Milk
	Porcine	0.75 μg kg^{-1}	Muscle
		2.0 μg kg^{-1}	Liver
		0.75 μg kg^{-1}	Kidney
Dexamethasone	Bovine, caprine, porcine,	0.75 μg kg^{-1}	Muscle
	Equidae	2.0 μg kg^{-1}	Liver
		0.75 μg kg^{-1}	Kidney
	Bovine, caprine	0.3 μg kg^{-1}	Milk
Methylprednisolone*	Bovine	10 μg kg^{-1}	Muscle
		10 μg kg^{-1}	Fat
		10 μg kg^{-1}	Liver
		10 μg kg^{-1}	Kidney
Prednisolone	Bovine	4.0 μg kg^{-1}	Muscle
		4.0 μg kg^{-1}	Fat
		10 μg kg^{-1}	Liver
		10 μg kg^{-1}	Kidney
		6.0 μg kg^{-1}	Milk
	Equidae	4.0 μg kg^{-1}	Muscle
		8.0 μg kg^{-1}	Fat
		6.0 μg kg^{-1}	Liver
		15 μg kg^{-1}	Kidney

* Not for use in animals from which milk is produced for human consumption.

The measures to monitor the residue control plan of glucocorticoids in livestock and derived food products are described in Council Directive 96/23/EC [43]. In Annex I, two groups of

substances (group A and B) are included, based on Commission Regulation No. 37/2010 [41]. In Italy and the Netherlands, glucocorticoids are classified as group A substances, comprising substances with hormonal effects, beta agonists and veterinary medicines that have been banned (Table 2 of Council Regulation No 37/2010)[41]. The other member states, including Belgium, regard them as group B substances, which holds the category of other pharmacologically active substances. To monitor the misuse and/or abuse of veterinary drugs in live animals and animal products, member states must draft and implement each year a National Residue Plan [43]. The analytical methods used for this control plan, must be in compliance with the criteria of Commission Decision 2002/657/EC [44].

In light of these national control plans, monitoring unauthorized administration of glucocorticoids in urine samples is of critical importance. Recently, the European Commission reported in the Commission Staff Working Document 'Implementation of national residue monitoring plans in the member states' (2009 - 2012), when focussing on glucocorticoids, an increasing occurrence of prednisolone residues (3.12 - 179.72 μg L^{-1}) in bovine urine samples without any direct evidence for unauthorized administration (Table 1.4.).

Table 1.4. Prevalence of non-compliant prednisolone bovine urine samples during the implementation of national residue monitoring plans in the member states [45][46][47][48]. In the Netherlands and Italy, corticosteroids are classed as group A3 substances, whereas other member states classify them as B2f.

Year	Substance group	Number of non-compliant results	Number of samples analysed	Member States reporting non-compliant results	% positive
2012	A3	4	3036	IT	0.10
	B2f	3	411	BE	0.73
	B2f	3	418	FR	0.72
	B2f	1	18	RO	5.55
2011	A3	7	3336	IT	0.20
	B2f	1	498	BE	0.30
2010	A3	2	3590	IT	0.06
	B2f	3	275	BE	1.10
	B2f	1	424	FR	0.24
2009	A3	3	2939	IT	0.10
	B2f	1	2410	BE	0.04
	B2f	5	206	ES	2.43
	B2f	3	428	FR	0.70

These findings, in essence originating from the increased sensitivity of analytical detection methods, have raised many questions. Several hypotheses concerning the origin of this low level prednisolone, have been suggested, i.e. prednisolone could be generated by physiologic metabolic processes under influence of stress, resulting in higher cortisol levels (during transport and slaughtering) [49][50][51] or by faecal microbial contamination of urine [52]. A well-known example of such a process is the microbial transformation of testosterone to β-boldenone by a Δ^1-steroid-dehydrogenase (Δ^1-SDH) [53][54]. Just like the anabolic androgenic steroid testosterone, cortisol and cortisone enclose a 3-oxo-4-ene structure, which is possibly the target of Δ^1-SDH for the 1,2-dehydrogenation. This reaction could result in the formation of prednisolone and prednisone, respectively (Figure 1.6.) [55].

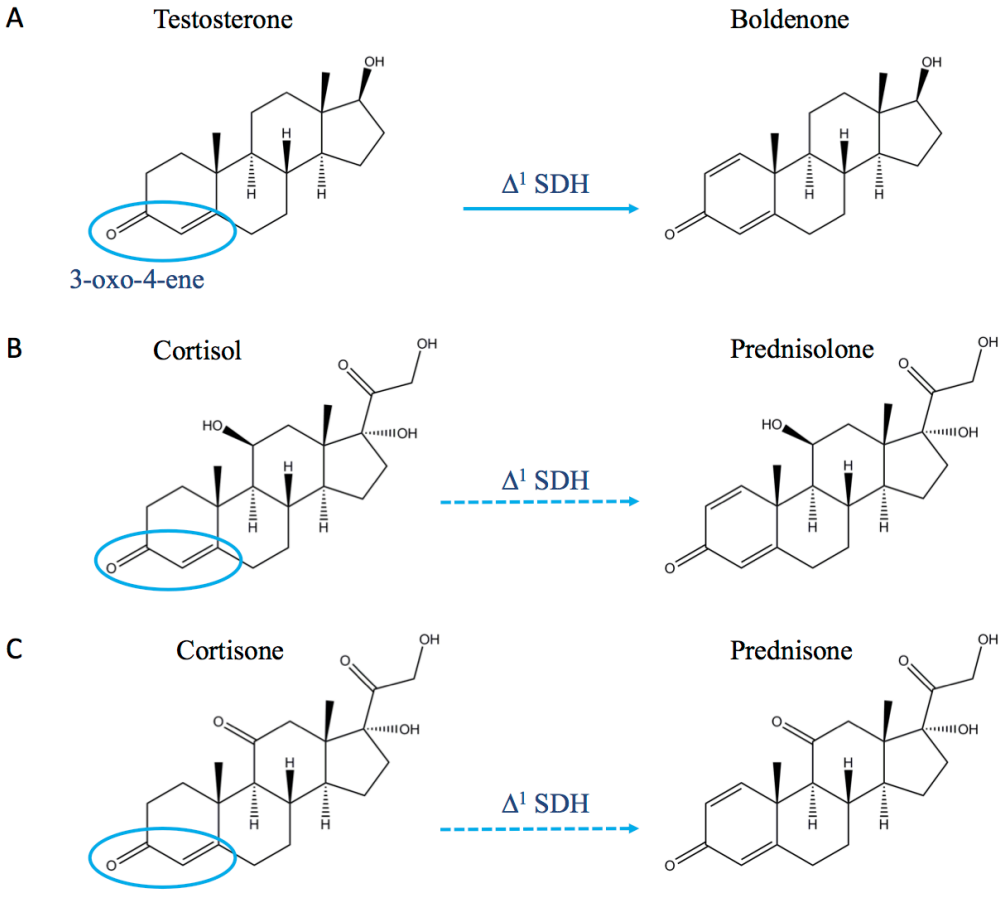

Figure 1.6. The microbial conversion of steroids of testosterone to β-boldenone (A) and possibly of cortisol to prednisolone (B) and cortisone to prednisone (C) by Δ^1-SDH (after Bredehöft *et al.*, 2012) [51].

Recent *in vitro* incubation experiments of cortisol with bovine S9 liver enzymes showed a significant decrease of cortisol together with the formation of prednisolone within six hours [56].

To account for potential other origins than unauthorized treatment, the European Union Reference Laboratories suggested a threshold level for prednisolone in bovine urine based on the following calculation: the average prednisolone concentration of 100 urine samples + three times the standard deviation [56]. Based on the results in literature, a threshold level for prednisolone of 5 µg L^{-1} was proposed [57][58][59].

2. Analysis of glucocorticoids in urine and faeces

In order to provide the consumer residue free food products of animal origin, proper analytical approaches for detecting glucocorticoid residues in edible or biological matrices are a necessity [43]. However, this is seen as an analytical challenge. After all, glucocorticoid residues may be present at very low concentrations disguised in a background of abundant primary and secondary metabolites [23]. Besides, chromatographic separation of these compounds is not straightforward due to their similar chemical configurations and chromatographic behavior [60][61]. As a consequence, the comprehensive analysis of glucocorticoids entails the use of a highly efficient extraction procedure and an analytical platform that is both sensitive and selective.

2.1. Sample preparation

Prior to the analysis, extensive clean-up steps have been employed in the majority of the reported analytical strategies to enable determination of glucocorticoids in urine and faeces. This is an absolute requirement for eliminating interfering substances, enriching glucocorticoids and performing solvent switching to the desired solvent conditions used for detection. This should all happen preferably without causing unwanted chemical degradation of metabolites.

Sample preparation procedures often start with hydrolysis of the present conjugated glucocorticoids by enzymatic or, to a lesser extent, chemical approaches (using acids like hydrochloric or sulphuric acid). Bacterial (*Escherichia coli*) and mollusc (*Helix pomatia*) enzymatic sources are most commonly used for this purpose. The bacterial sources only contain β-glucuronidase activity to cleave the glucuronide conjugates, whereas the mollusc sources contain both β-glucuronidase and sulfatase activities [62][63]. However, it was shown that deconjugation is dispensable, as prednisolone and cortisone are almost exclusively present in

bovine urine and faeces in their free form [23]. Moreover, the deconjugation process itself can negatively influence the accuracy of analyte measurement by inducing unwanted transformations i.e. androstenedione to testosterone [64][65][66][67].

Extraction of glucocorticoids can be performed by liquid-liquid extraction in which compounds are secluded on the basis of their relative solubility in two different immiscible liquids [68]. Therefore various organic solvents including diethylether, tert-butyl-methyl ether, ethyl acetate, methanol and hexane are used [35][69][70][71][72]. This extraction is usually repeated several times to improve the efficiency [74]. Depending on the complexity of the matrix, the obtained extract may be evaporated to dryness under a gentle stream of nitrogen [40] or further purified by solid-phase extraction [60]. This is a sample clean-up technique in which glucocorticoids are trapped on a solid-phase extraction column, in function of their physical and chemical properties while the interfering compounds are eluted or vice versa. The main separation modes (and sorbents) include anion-exchange (Sephadex, Ammonium) [23][75][76], reverse-phase (C18, Oasis HLB, Strata-X) [77][78][79] and mixed mode (Oasis MAX) [60] cartridges.

A particular type of extraction that has been applied for glucocorticoids as well is the QuEChERS procedure, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe [80]. This type of extraction involves initial single-phase extraction of samples with acetonitrile, followed by liquid-liquid partitioning under the influence of anhydrous $MgSO_4$. Removal of residual water and clean-up are performed simultaneously by using a rapid procedure called dispersive solid-phase extraction, in which anhydrous $MgSO_4$ and a sorbent (e.g. primary secondary amine) removes water and polar compounds [81].

2.2. Analytical detection approaches

For the detection of glucocorticoids different analytical methods exist, e.g. immunoassay, chromatography coupled to different types of detectors relying on ultraviolet, fluorescence or mass spectrometry [82]. For accurate quantification however, the most applied technology is mass spectrometric detection hyphenated to a separation technique. First the mass spectrometer was coupled to gas chromatography. But more recently, due to advances in atmospheric pressure ionization interfaces such as electrospray and atmospheric chemical

ionization, it is possible to couple a mass spectrometer to liquid chromatography systems [83]. In this regard, the key concept of each technology is given.

2.2.1. Immunoassays

Enzyme immunoassays (EIA) and radioimmunoassays (RIA) are based on an antibody that binds a specific compound. In response to binding, a measurable signal is produced due to an enzymatic reaction (EIA) or radioactive isotopes bound at the antibody (RIA). These methods are considered as easy and accessible screening methods, enabling the detection of several faecal glucocorticoid metabolites e.g. 11,17-dioxoandrostanes, cortisol-3-(carboxymethyl)oxime, 11β-hydroxyetiocholanolone [84][85][86] or free cortisol in urine [87].

These methodologies can be highly sensitive but are susceptible to cross-reactivity of the specific antibody with other structurally related endogenous and exogenous steroids. It has been demonstrated that RIA, especially those based on direct assays, often overestimate true steroid values [88][89]. Another limitation of the RIA techniques is the impossibility of using an internal standard to monitor the recovery during extraction. Comparison between RIA, EIA and chromatographic methods clearly indicates the superiority of chromatographic-based methods towards quantification [87].

2.2.2. Chromatographic separation coupled to mass spectrometric detection

Chromatography separates a mixture into its components by interaction between two immiscible phases, of which the mobile phase moves along the stationary phase. Due to the similar chemical configuration and chromatographic behaviour of glucocorticoids, chromatographic separation is not straightforward. Different types of chromatography can be conducted for this purpose, however in this dissertation only the differentiation between gas and liquid chromatography will be discussed.

2.2.2.1. Gas chromatography

During the 1990s, gas chromatography coupled to mass spectrometry was considered the gold standard for steroid quantitation [90]. It is a sensitive, robust and therefore a suitable technique for the detection of these hormones, despite the lengthy sample preparation and the need for derivatization. Silylation, acylation or oxime/silylation are derivatization procedures enabled to

reduce the analyte polarity and thermal instability [91][92][93]. Nowadays, several laboratories still measure steroid concentration by GC-methods [94][95].

2.2.2.2. Liquid chromatography

In the mid 1990s, high-performance liquid chromatography (HPLC) has emerged as an effective alternative for the analysis of glucocorticoids [60][96]. It does present some limitations in terms of run time and chromatographic resolution. This limitation can be circumvented by means of ultra high-performance liquid chromatography (UHPLC) that enables faster separation of compounds in comparison to traditional LC, due to the use of columns with sub 2 µm particles. UHPLC provides improved speed of analysis, a better resolution, increased sensitivity and reduction of matrix effects [97].

To effectuate glucocorticoid separation, reversed phase (U)HPLC using silica based C18 material is most intensively used [77][78][98]. With regard to the mobile phase, solvents are mostly modifications of acetonitrile or methanol (Table 1.5.). A small percentage of formic acid or ammonium acetate is usually added to obtain the desired retention and improve compound separation [60][79][80]. Once the analytes have been separated over time, the liquid phase needs to be evaporated and ionised by a proper interface system.

Table 1.5. Selected confirmatory methods for detecting glucocorticoids in biological fluids using chromatographic separation followed by mass spectrometric detection [83].

Matrix	Column; mobile phase; flow rate	Detection mode	Ref.
Urine	BEH C18 column (2.1 mm×50 mm, 1.7 µm); 0.1% formic acid in H_2O/0.1% formic acid in ACN, at 0.35 mL/min	ESI positive	[70]
Plasma	Pursuit C-18 column (2.0 mm×150 mm, 5 µm); 0.5% formic acid in H_2O/ACN, at 0.20 mL/min	ESI positive	[99]
Plasma, Urine, Saliva, Plasma	BEH C-18 column (2.1 mm×50 mm, 1.7 µm); 2 mM ammonium acetate in water with 0.1% formic acid/2 mM ammonium acetate in methanol with 0.1% formic acid, at 0.40 mL/min	ESI positive	[78]
Urine	Halo C-18 column (2.1 mm×150 mm, 2.7 µm and 2.1 mm×100 mm, 2.7 µm); 0.1% formic acid in H_2O/0.1% formic acid in ACN, at 0.40 mL/min	ESI positive	[100]
Urine	Zorbax C-18 column (2.1 mm×50 mm, 1.8 µm); 0.1% acetic acid in H_2O/0.1% acetic acid in ACN, at 0.30 mL/min	ESI positive	[101]
Urine	Inertsil ODS-3 C18 column (50 mm×4.6 mm, 3 µm); 1% formic acid in H_2O/ACN, at 0.70 mL/min	ESI positive	[102]
Urine	Synergi C18 (250 mmx4.6. mm, 4 µm); 1% acetic acid in H_2O/ACN, at 0.80 mL/min	APCI negative	[50]

2.2.2.3 Interface systems

Various atmospheric pressure ionization (API) systems in both the positive and negative ion modes e.g. electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), enable ionization of glucocorticoids. Haneef *et al.* (2013) [83] presented an overview of the ionization techniques used for detecting glucocorticoids in biological fluids (Table 1.5.).

ESI is a soft ionization technique that generates gas phase ions from a typical liquid-phase (Figure 1.7.A). After passing the capillary needle on which a strong electrostatic field is applied, the LC eluent is nebulized into a chamber at atmospheric pressure. The heated drying gas evaporates the solvent into droplets, which results in increasing charge concentration in the droplets. Eventually, the repulsive force between ions with the same charges exceeds the cohesive forces and non-fragmented ions are ejected into the gas phase. These ions are attracted to and thus proceed to the mass analyser. The heated electrospray ionization (HESI) interface has increased capacity for mobile phase desolvation, due to thermal desolvation assistance in the capillary needle [103].

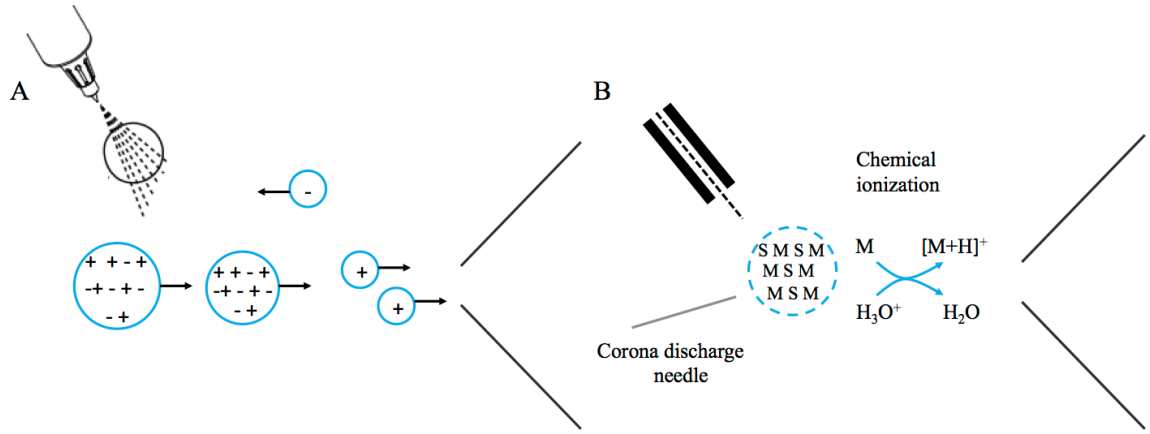

Figure 1.7. Schematic of electrospray ionisation (A) and chemical ionisation (after Thermo Fisher Scientific, 2012).

APCI in positive and negative mode was proven to be suitable for the ionisation of corticosteroids in urine [90] (Figure 1.7.B). With APCI, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube before interacting with the corona discharge creating ions. The LC eluent is sprayed through a heated (> 250 °C) vaporizer at atmospheric pressure that vaporizes the liquid. The resulting gas phase solvent molecules are subsequently ionised by electrons discharged from a corona needle and transfer their charge to

the analyte molecules through chemical reactions. Positive ions are formed through proton transfer, while negative ions are formed through electron transfer or proton loss [103].

2.2.2.4 Mass spectrometry

One of the most applied mass spectrometric techniques in hormone and veterinary drug residue analysis, is quadrupole tandem mass spectrometry (QqQ-MS/MS). This type of instrument achieves high sensitivity, selectivity and reproducibility through the selected reaction-monitoring (SRM) mode [23][78][98]. Nevertheless, this technology presents limitations with regard to the limited number of compounds that can be analysed in one run and only in advance defined analytes can be detected since there is no possibility of retrospective data analysis. Because of these limitations, there is currently a trend towards full-scan approaches using high-resolution mass spectrometry (HRMS) [80]. Within this context, Fourier Transform Ion Cyclotron Resonance (FT-ICR), Time of Flight (ToF), and Fourier Transform Orbitrap have emerged as key technologies [104].

These instruments are characterised by *high mass resolution*, which is the ability to distinguish between narrow mass spectral peaks. This parameter can be interpreted in two ways. In case of the valley-based definition, mass resolution is the ratio between the mass of the second peak and the mass difference between peaks. Alternatively, mass resolution may also be determined based on an isolated peak whereby the mass difference now relates to the peak width at a specified peak height. This peak width based approach is usually applied at peak half maximum (full width at half maximum, FWHM). Besides high resolution, these instruments provide a *full-scan mass spectrum* of theoretically all analytes that were introduced into and ionized by the ion source [105][106]. This makes retrospective evaluation of acquired data for non-"a priori" selected compounds possible by reconstructing any desired ion chromatogram [107]. In addition, these mass analyzers provide *high mass accuracy*, which is indicative for the difference between the theoretical and measured *m/z*-vale and expressed in millimass units (mmu) or in parts per million (ppm) [108].

The best results in terms of accuracy (< 1.5 ppm) and mass resolving power (up to 1,000,000) are achieved with FR-ICR instruments. However, these instruments request a time-consuming signal optimization, long acquisition time and are expensive, which makes them less popular in

comparison to Orbitrap and ToF based instruments [109]. ToF analyzers present a mass resolution up to 60,000 FWHM and mass accuracy below 2 ppm but cover only a limited dynamic range over which accurate measurements of mass can be made [96]. The latter is in contrast with Orbitrap mass analyzers. Besides, these obtain a mass accuracy of < 3 ppm and a resolution up to 240,000 FWHM. Its full-scan speed (10 Hz) allows fast, reproducible and reliable analytical results [104][110].

The operating principle of the Orbitrap system is mainly founded on its ability to trap ions (C-trap) and allow them to start spinning around a central electrode (Figure 1.8.). The C-trap, a curves radio frequency-only quadrupole ion trap, injects a compact package of ions into the orbitrap mass analyser. As an ion packet enters, the electric field is switched on and attracts the ions to the central electrode. If the ions are sufficiently fast, they will not plunge onto the electrode but rotate around the central axis while performing axial oscillation. The frequency of this oscillation is proportional to the mass-over-charge (*m/z*) ratio and is detected and processed by fast Fourier transform to obtain a spectrum. Structural information can be generated in a non-selective manner (all ions fragmentation) by using a High Energy Collisional Dissociation (HCD) cell without precursor ion selection [111][112].

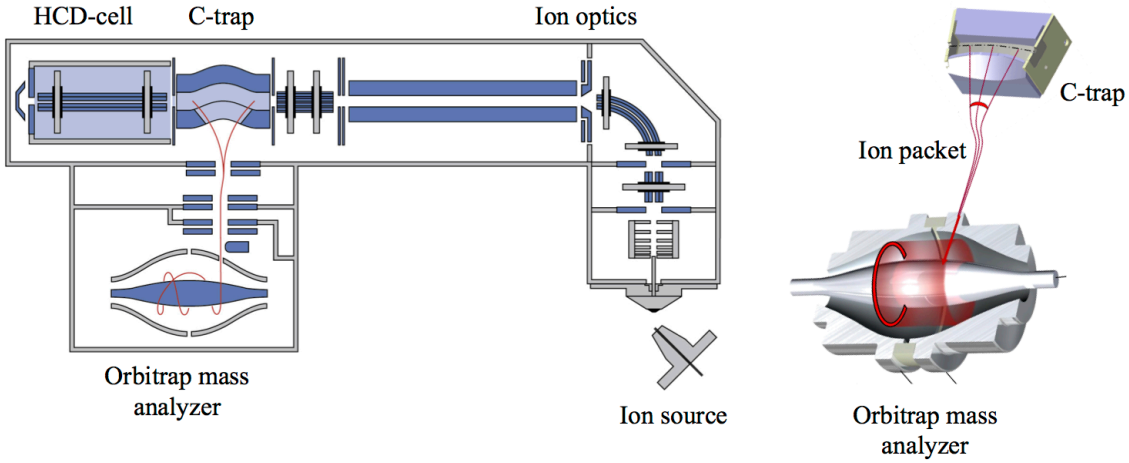

Figure 1.8. Schematic representation of a bench-top Exactive™ Orbitrap mass spectrometer and the orbitrap mass analyser element.

The newest innovation in LC-MS/MS techniques are the so called hybrid mass spectrometers. These combine the full-scan HRMS with a linear ion trap or a quadrupole mass filter to acquire structural information in a selective manner [104][113].

The emergence of hybrid MS, including LTQ-Orbitrap, Q-Exactive and Q-ToF systems, has promoted the development of automated data acquisition in the MS/MS mode to maximise throughput capabilities and the information obtained from a single analytical run [114][115][116] such as data-dependent acquisition (DDA) and data-independent acquisition (DIA) [117]. In DDA MS/MS spectra are acquired if selected criteria (e.g. threshold, charge of the detected compound and dynamic exclusion list) are met in a survey scan, which is generally based on a full-scan to ensure that the process is non-discriminative [118]. This approach has enabled the systematic selection and fragmentation of the base peak ion of the full-scan spectrum for the acquisition of specific MS/MS spectra. The same approach can then be extended to the second and third most abundant ions, although this is limited by the duty cycle that is necessary to acquire the MS/MS dependent scan [119]. Unlike DDA, DIA is not biased towards the detection of the most abundant ions in a full-scan spectrum because it does not use a selection step prior to fragmentation. For this purpose, any *m/z* contained in a range of interest is fragmented either by simultaneously broadbanding all ions (entering the MS at a single chromatographic time point) (MS^E and AIF, all ion fragmentation) or by multiplexing the full *m/z* range into smaller *m/z* isolated windows. Both MSX-DIA, multiplexed MS/MS data-independent acquisition and SWATH, Sequential Window Acquisition of all Theoretical mass spectra, are based on the fragmentation of all ions by dividing the *m/z* window into smaller mass ranges [117][120]. Although this approach results in more complex datasets, multiple advantages have been achieved with these strategies, including comprehensive qualitative/quantitative enquiries of samples with high specificity and the possibility of retrospectively mining data because all *m/z* are fragmented within the LC time frame [121].

3. Discriminating endogenous from exogenous prednisolone: metabolomics

The analytical methods used for monitoring the misuse and/or abuse of veterinary drugs in food producing animals, are generally targeted-oriented procedures [122]. An inherent limitation is the inability to detect residues such as novel unauthorized administered growth promoters or the lack to differentiate between an endogenous or exogenous origin of a certain compound. A

well-known example of the latter is the relatively recent discovered endogenous formation of boldenone [123][124]. More recently, a higher frequency of prednisolone levels in bovine urine has been noticed, which could not be directly related to fraudulent use of prednisolone. As such, questions have risen about the origin of this compound. Due to the similar endogenous and exogenous urinary concentrations of prednisolone, at present, no decisive strategy has been established to discriminate between endogenous and exogenous prednisolone (Table 1.6.). This makes it necessary to consider novel analytical strategies.

Table 1.6. Overview of the reported urinary prednisolone concentrations upon exogenous administration and following endogenous detection.

	Administration/ Origin	**Doses**	**CCα (μg L^{-1})**	**Concentration (μg L^{-1})**
Exogenous administration				
Cannizzo *et al*. (2011) [40]	Prednisolone	15 mg/day PO during 30 days	0.67	0.51 - 0.68
	Prednisolone	30 mg/day PO during 35 days	0.67	0.27 - 1.18
Nebbia *et al.* (2014) [33]	Prednisolone acetate	2 x 0.4 - 0.5 mg/kg in 48h	0.05	40 - 72
Endogenous detection				
Pompa *et al.* (2011) [49]	ACTH treatment	2 x 2 mg in 24h IM	0.05	0.69 - 4.08
Ferranti *et al.* (2011) [125]	Control group	N/A	0.40	0.50 - 0.70
	Slaughterhouse	N/A	0.40	0.40 - 1.50
Vincenti *et al.* (2011) [51]	Farm	N/A	0.70	Not detected
Ferranti *et al.* (2013) [50]	Control group	N/A	0.30	0.60 - 0.80
	Slaughterhouse	N/A	0.30	0.70 - 6.20
Bertocchi *et al.* (2013) [126]	Farm	N/A	0.40	1.06 ± 0.65
	Slaughterhouse	N/A	0.40	0.84 ± 0.45
de Rijke *et al.* (2014) [56]	Farm	N/A	0.20	0.11 - 0.90
	Slaughterhouse	N/A	0.20	0.11 - 2.04

N/A: Not applicable

A prominent trend in analytical detection methods is the shift from targeted oriented approaches towards accurate mass full-scan HRMS [106], allowing the detection of non-a priori selected compounds. This shift allows differentiating exogenous and endogenous compounds on another level, i.e. comparing complete urinary metabolite profiles.

Urinary steroid profiles provide quantitative information on the steroid biosynthetic and catabolic pathways and can be essential for identification of inborn errors or other disorders with altered steroid secretion [127]. Besides, administration of steroid hormones may alter

endogenous steroids profiles as well as related metabolite levels in urine as a consequence of direct or indirect biological response. In this regard, strategies based on the detection of changes in the metabolome are promising approaches in human antidoping control [20][128] or to highlight growth-promoting practices for animal fattening purposes [94][129][130][131].

An important factor before relying on a metabolomics approach is the availability of a suited matrix, i.e. a matrix that contains a large range of potential interesting metabolites and reflects the current metabolic state of the host [129]. In this context urine is the matrix of choice, since it fulfils these conditions and can be easily, non-invasively, obtained.

3.1. Metabolomics

Metabolomics is defined as the qualitative and quantitative analysis of small metabolites (< 1500 Da) in a specific biological system at a certain time point, under specific conditions [132]. It facilitates identification of patterns or biomarkers correlated to prednisolone administration [133]. It is useful whenever an assessment of changes in metabolite levels is important.

3.2. General strategy for metabolomics analysis

Within the metabolomics, two major approaches can be discerned: metabolite profiling and metabolite fingerprinting. These strategies differ in many aspects including the level of quantitation, complexity of sample preparation, experimental accuracy and precision and number of metabolites detected.

Metabolite profiling is the oldest and most established approach and is considered the precursor of metabolomics [134]. It is the analysis of a group of known metabolites either related to a specific metabolic pathway or a class of compounds e.g. steroids [135]. These methods are developed with the application of authentic chemical standards, offer medium throughput and a semi-quantitative output [94]. The process of the acquired data can start immediately following data analysis, since the chemical identity of the metabolites is known [136]

Metabolic fingerprinting does not attempt to identify or precisely quantify all metabolites in the analysed sample. Rather, it considers a total fingerprint, as a unique pattern reflecting the metabolic activity at that time in e.g. a urine sample. Pattern recognition tools are used to classify the fingerprints and identify the specific features of the fingerprint that are characteristic

for each pattern [137]. Metabolic fingerprinting is most useful in biomarker discovery and diagnostics [138].

After a biomarker is uncovered, targeted analysis may be performed to focus on the quantitative analysis of a predefined number of known metabolites related to an uncovered metabolic pathway or class of compounds. For this, a specific metabolite extraction, separation and detection will be developed and optimized in order to achieve very low limits of detection and high throughput [139]. The chemical identification and structural elucidation of the newly discovered compounds is a labour-intensive step that follows data acquisition and must occur before biological interpretation is possible. The major limitation of the targeted analysis of the newly defined metabolites, is that it requires the compounds available in purified standards [137].

3.3. General workflow for metabolomic analysis

The workflow that has been created for untargeted metabolomic studies consists of multiple steps (Figure 1.9.).

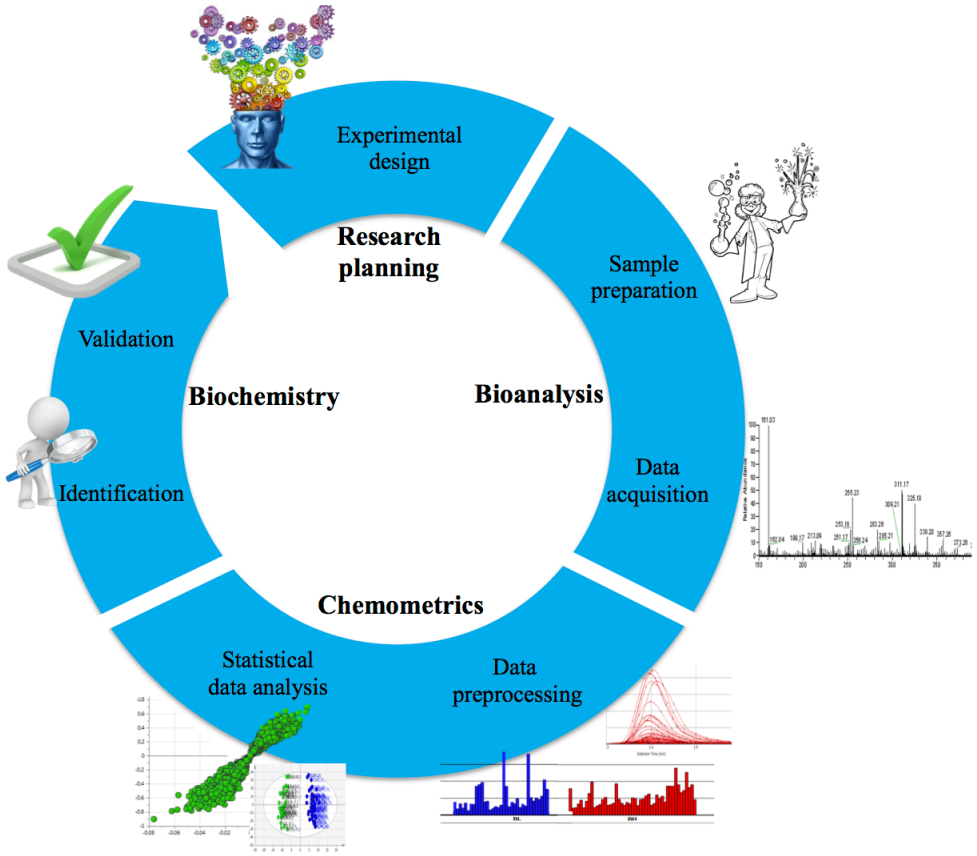

Figure 1.9. Typical workflow for untargeted metabolomic studies including research planning, bioanalysis, chemometrics and biochemistry.

The comprehensive investigation of the metabolome is being hampered by its enormous complexity and dynamics. In order to extract the maximum amount of information, secure planning of the *experimental design* is mandatory for the success of these types of investigations [140]. Therefore it is important to consider and carefully plan sample collection, storage and stability of the matrix and compounds, intra-individual variation due to environmental factors such as diurnal variation and stress, inter-individual variations due to genetic factors etc. [141].

In metabolomics, *sample preparation* may have a major impact on the obtained results. The goal is extracting as many metabolites as possible without causing chemical degradation while reducing the matrix effects and ion suppression. The main steps in most sample preparation procedures include metabolite extraction with an appropriate solvent, and a procedure to enrich the metabolite content in order to achieve sensitive detection limits [135][142]. For metabolic fingerprinting, a sample preparation procedure isn't expected to be highly selective but has to extract a broad range of compound classes in a robust and reproducible manner [130].

Two analytical platforms are by far the most used for *data acquisition* within metabolomic analysis: mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The great potential of NMR lies in its non-discriminatory, non-destructive nature, while it provides unique structural information. Although NMR requires limited sample preparation, it will only detect medium to high abundant metabolites [143][144]. MS-based approaches currently yield higher sensitivity than NMR when analysing minimal amounts of complex mixtures [145]. In particular the use of high and ultra high-resolution mass spectrometry greatly improves analytical performance and offers the best combination of selectivity and sensitivity. Mass analyzers working in tandem or hybrid configuration can aid metabolite identification by acquiring highly resolved and accurate MS/MS spectra [110][146].

During *data mining*, the acquired metabolomics data is converted into valuable and meaningful information in successive steps. Data pre-processing constitutes the initial step in data handling and its goal is to extract all the relevant information from the raw data and summarize them. This procedure includes steps such as noise filtering, data binning, peak detection and chromatographic alignment [130]. Data pre-treatment e.g. normalization, scaling, etc. is used to correct or reduce unwanted technical variation, which could limit the interpretability of

metabolomics data. Statistical modelling can highlight and identify underlying similarities and differences in huge data sets and seek fingerprints or patterns that allow sample discrimination or biomarker discovery [147][148].

Currently, the process of metabolite *identification* in untargeted metabolomic studies is seen as the bottleneck in deriving biological knowledge from metabolomic studies. Nevertheless, mass spectrometry can provide a wealth of structural information e.g. *m/z*-ratios, relative isotope abundance and fragmentation patterns. The comparison of this experimental data to mass spectral libraries can provide reliable information (Table 1.7.) [132][149] but it is unrealistic for all metabolites to be incorporated into mass spectral libraries. Even when these data are available, the transferability of libraries between instruments must be considered [136][150]. Besides, the lack of suitable reference standards to be purchased makes that the identification can't be performed at the highest level [138][151].

Table 1.7. Overview of available web-resources useful in metabolomics research [150][152].

Database	Type	Link
Comprehensive Metabolomic Databases		
HMDB	Human Metabolome Database	http://www.hmdb.ca
BiGG	Biochemical Genetic and Genomic knowledge base of large scale metabolic reconstructions	http://bigg.ucsd.edu
SetupX	Developed by the Fiehn laboratory at UC Davis, is a web-based metabolomics LIMS.	http://fiehnlab.ucdavis.edu/projects/binbase_setupx
BinBase	A GC-TOF metabolomic database	http://fiehnlab.ucdavis.edu/projects/binbase_setupx
MetaboLights database	Database for metabolomics experiments and derived information	http://www.ebi.ac.uk/metabolights/index
Urine Metabolome database	Database containing detailed information about molecule metabolites found in human urine	http://www.urinemetabolome.ca
BMDB	Bovine Metabolome Database	http://www.cowmetdb.ca/cgi-bin/browse.cgi
Metabolic Pathway Databases		
KEGG	Kyoto Encyclopedia of Genes and Genomes	http://www.genome.jp/kegg
MetaCyc	Metabolic pathway database	http://metacyc.org
HumanCyc	Bioinformatics database that describes the human metabolic pathways and the human genome	http://humancyc.org
Compound or Compound-Specific Databases		
PubChem	Freely available database of chemical structures of small organic molecules and information on their biological activities	http://pubchem.ncbi.nlm.nih.gov
CheEBI	Chemical Entities of Biological Interest	http://www.ebi.ac.uk/chebi/
ChemSpider	Aggregated database of organic molecules containing more than	http://www.chemspider.com

	20 million compounds from many different providers	
IIMDB	In Vivo/In Silico Metabolites Database	http://metabolomics.pharm.uconn.edu/iimdb/
Drug Databases		
DrugBank	Blended bioinformatics and cheminformatics resource that combines detailed drug data with comprehensive drug target information	http://www.drugbank.ca
TTD	Therapeutic Target Database	http://xin.cz3.nus.edu.sg/group/ttd/ttd.asp
STITCH	Search tool for interactions of chemicals	http://stitch.embl.de
SuperTarget	Database that contains a core dataset of about 7300 drug-target relations of which 4900 interactions have been subjected to a more extensive manual annotation effort.	http://bioinf-apache.charite.de/supertarget_v2/
Spectral Databases		
BMRB	Biological Magnetic Resonance Databank	http://www.bmrb.wisc.edu/metabolomics/
MMCD	Madison Metabolomics Consortium Database	http://mmcd.nmrfam.wisc.edu
MassBank	A mass spectral database of experimentally acquired high resolution MS spectra of metabolites	http://www.massbank.jp
Golm Metabolome Database	Provides public access to custom GC/MS libraries which are stored as Mass Spectral (MS) and Retention Time Index (RI) Libraries (MSRI)	http://gmd.mpimp-golm.mpg.de
Metlin	Metabolite Database	https://metlin.scripps.edu/index.php
Fiehn GC-MS Database	Contains GC/MS data (spectra and retention indices) collected by the Fiehn laboratory.	http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/
BML-NMR	Birmingham Metabolite Library Nuclear Magnetic Resonance	http://www.bml-nmr.org
mzCloud	Collection of high resolution/accurate mass spectral trees using a new third generation spectra correlation algorithm	https://www.mzcloud.org

Besides the web-based databases, other tools allow reduction of the metabolite search space to a single or small number of metabolites to achieve putative annotation. This includes collation of data for unidentified metabolites (e.g. accurate measurements of *m/z*, fragmentation mass spectra related to chemical structure, the application of chemical and biological knowledge, experimental isotope-based studies) and the development of *in silico* tools to predict mass spectral and chromatographic properties [136][153].

It is necessary to *validate* the performance and usefulness of biomarkers. Hereby, a distinction should be made between analytical method validation and clinical qualification. Validation is defined as the process of assessing the biomarker and its measurement performance characteristics, and determining the range of conditions under which the biomarker will give reproducible and accurate data [154][155][156]. While clinical qualification is the evidentiary process of linking a biomarker with biological processes and clinical endpoints [157].

4. Conceptual framework of this study

The European Commission reported more non-compliant urine samples for prednisolone in the past few years in their annual Commission Staff Working Document on the implementation of the established national glucocorticoid monitoring plans in the different member states, without any direct evidence of unauthorized use. These findings raised many questions about the origin of this prednisolone. At the start of this PhD project, a mechanistically explanation or a direct discrimination between endogenous and exogenous prednisolone had not yet been established.

In literature a few hypotheses had been put forward, which were further investigated in this study. A first is the possible conversion of natural glucocorticoids into prednisolone during inappropriate storage of the samples of interest. Therefore, this study aimed at deepening the knowledge about the long-term stability of natural and synthetic glucocorticoids in two important matrices from a control perspective i.e. urine and faeces. To this end, analytical methods for the accurate and specific determination of natural and synthetic glucocorticoids in urine and faeces were developed and validated according to CD 2002/657/EC [44] (**Chapter II and Chapter III**).

A second hypothesis states that prednisolone could be generated under the influence of stress, since most of the prednisolone positive bovine urine samples were collected at the slaughterhouse so far. To take into account this possible endogenous formation of prednisolone, the European Reference Laboratories have suggested a threshold level for prednisolone in bovine urine of 5 $\mu g\ L^{-1}$. To investigate this hypothesis and the related threshold level, bovine urinary levels of cortisol, prednisolone and their main phase I and II metabolites were evaluated under conditions differing in degree of stress: at the farm, after slaughter and upon administration of a synthetic analogue of adrenocorticotropic hormone. Besides, the urinary metabolic fingerprint of the different urine batches was evaluated and differentiating metabolites were assigned that have a key role in the urinary metabolome in response to stress. This could be a powerful tool to classify an unknown bovine urine sample and offer information about the animal's individual condition (**Chapter IV**).

In literature, the use of prednisolone/cortisol urinary concentration ratios and the analysis of 20β-dihydroprednisolone were suggested as potential screening tools to confirm the origin of prednisolone. Little is however known about the pharmacokinetics of the known main metabolites of prednisolone. Therefore, the pharmacokinetic and urinary excretion profile of prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone were assessed during a growth-promoting and therapeutic prednisolone treatment on the one hand and during pharmacologically-induced increase of cortisol on the other hand. In addition, the biotransformation of cortisol was investigated by profiling urinary glucocorticoid metabolites during these treatments (**Chapter V**).

Because the need exists for a specific biomarker that has the ability to discriminate endogenous formation from exogenous administration of the synthetic glucocorticoid prednisolone, a strategy of metabolic fingerprinting was implemented to assess potential metabolite differences in the urine of cows, exposed to a growth-promoting and therapeutic prednisolone treatment. This encompassed metabolite discovery, but also evaluation of classification performance by defining the sensitivity, specificity, urinary excretion kinetics and selectivity (**Chapter VI**).

Finally, **Chapter VII** summarizes and reflects on the eliciting findings of the different research chapters and some conclusions are formulated.

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CHAPTER II:

A VALIDATED ANALYTICAL METHOD TO STUDY THE LONG-TERM STABILITY OF NATURAL AND SYNTHETIC GLUCOCORTICOIDS IN LIVESTOCK URINE USING ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO ORBITRAP-HIGH RESOLUTION MASS SPECTROMETRY

Adapted from:

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Abstract

Due to their growth-promoting effects, the use of synthetic glucocorticoids is strictly regulated in the European Union (Council Directive 2003/74/EC). In the frame of the national control plans, which should ensure the absence of residues in food products of animal origin, in recent years, a higher frequency of prednisolone positive bovine urines has been observed. This has raised questions with respect to the stability of natural corticoids in the respective urine samples and their potential to be transformed into synthetic analogues. In this study, an ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) methodology was developed to examine the stability of glucocorticoids in bovine urine under various storage conditions (up to 20 weeks) and to define suitable conditions for sample handling and storage, using an Orbitrap Exactive^{TM}. To this end, an extraction procedure was optimized using a Plackett-Burman experimental design to determine the key conditions for optimal extraction of glucocorticoids from urine. Next, the analytical method was successfully validated according to the guidelines of CD 2002/657/EC. Decision limits and detection capabilities for prednisolone, prednisone and methylprednisolone ranged, respectively, from 0.1 to 0.5 µg L^{-1} and from 0.3-0.8 µg L^{-1}. For the natural glucocorticoids limits of detection and limits of quantification for dihydrocortisone, cortisol and cortisone ranged, respectively, from 0.1 to 0.2 µg L^{-1} and from 0.3 to 0.8 µg L^{-1}.

The stability study demonstrated that filter-sterilization of urine, storage at -80 °C, and acidic conditions (pH 3) were optimal for preservation of glucocorticoids in urine and able to significantly limit degradation up to 20 weeks.

1. Introduction

The well-known anti-inflammatory properties of the natural glucocorticoid cortisol, has led to the development of synthetic glucocorticoid analogues, which exert even higher anti-inflammatory activities [1]. Nowadays, the commonly used therapeutic glucocorticoid drugs in veterinary medicine comprise betamethasone, dexamethasone, methylprednisolone and prednisolone, with prednisone as prodrug. Beside the anti-inflammatory properties, these drugs also induce body weight gain in production animals by improving feed intake and lowering feed conversion. However, due to their growth-promoting effects and potential consumer's health risks [2][3], the use of glucocorticoids in livestock has been strongly restricted within the European Union [4].

The analysis of glucocorticoids, which is of critical importance in light of the national control plans within the EU, remains, however, a challenging task. After all, glucocorticoid residues are typically present in urine at very low concentrations in a background of a wide range of more abundant primary and secondary metabolites [5]. Besides, chromatographic separation of these compounds is not straightforward due to their similar chemical configurations and chromatographic behaviour [6][7]. Because of the identical molecular masses of cortisone and prednisolone, co-elution may result in a loss of selectivity. Indeed, the natural prevalence of the glucocorticoids cortisol and cortisone in urine at low concentrations may hamper the analysis of prednisolone and prednisone [5]. Nevertheless liquid chromatography coupled to mass spectrometry has been proven suitable to enable sensitive detection of glucocorticoids in urine [6][8][9][10]. Because conventional liquid chromatography presents some limitations in terms of a longer run time and lower chromatographic resolution, ultra-high performance liquid chromatography (UHPLC) using columns with sub 2 µm particles, is more commonly used these days [11][12][13]. Due to the very narrow peaks produced by UHPLC, a compatible fast scanning MS device is required. The most applied mass spectrometric technique in hormone and veterinary drug residue analysis is quadrupole tandem mass spectrometry (QqQ-MS/MS) [14], relying on the high sensitivity and selectivity of the selected reaction-monitoring (SRM) mode of QqQ-MS/MS. This technology has certain limitations since there is no possibility of post-acquisition re-interrogation of data and only a limited number of compounds can be measured

within one run (max. 100 - 120 using timed SRM). Also the screening for unidentified and unknown compounds is not possible because the fragmentation behaviour of the compound is unknown [15][16]. Because of these limitations, there is currently a trend towards full scan high resolution MS analysis using amongst others ToF (time of flight) instruments, with mass deviations below 5 parts per million (ppm) and resolutions of about 15,000 full width at half maximum (FWHM) [11][15]. However, in complex matrices this resolution is inadequate for accurate mass measurements. One of the most attractive and relatively new techniques is the Fourier Transform Orbitrap mass spectrometric technology with a resolving power up to 100,000 FWHM and a precise mass deviation below 2 ppm [15], allowing fast, reproducible and reliable analytical results for multiple residue analysis [12].

Prior to HRMS analysis, it is common to apply a generic extraction to allow as much as possible relevant analytes to be retained in the extract, but at the same time remove potential matrix interferences. The Plackett-Burman experimental design is a highly efficient and useful tool to screen for the main variables within a large number of variables that may affect the extraction yield [17][18]. This time saving approach, providing the opportunity to identify the optimal conditions for extraction of a certain number of analytes from a matrix by evaluating a large number of variables with a minimum of experiments. It also permits estimation of random error variability and testing the statistical significance of the variables [17][19].

The European Commission reported in 2012 in the Commission Staff Working Document on the implementation of national residue monitoring plans in the member states in 2010 that 0.14% of the bovine urine samples were non-compliant for prednisolone (3.12 µg L^{-1} - 179.72 µg L^{-1}) [20]. This high frequency of bovine urine samples found positive for prednisolone without any direct evidence for unauthorized use, has raised many questions [8][9][10][21]. A possible hypothesis is the conversion of the natural glucocorticoid cortisol into prednisolone, during inappropriate storage [9][10]. The prevalence of faecal microbiota in urine may indeed alter the endogenous concentration of steroid hormones [22][23]. A well-known example of such a process is the microbial transformation of testosteron to boldenone by a Δ^1-dehydrogenation [24][25]. Due to structural similarities, similar reactions may be expected from cortisol to prednisolone and cortisone to prednisone [23]. Research with respect to the long-term stability of these

compounds and possible changes occurring during storage is, however, scarce and mainly targeted oriented.

Therefore, the present study examined the changes in glucocorticoid concentrations of bovine urine samples during a long-term storage experiment, in which the effect of different storage conditions such as pH and temperature were considered. Additionally, the preservation under aerobe and anaerobe environments, as well as the contamination with faecal bacteria, was evaluated. Furthermore, this stability study included the determination of losses during multiple freeze-thaw cycles. The compounds of interest were the natural glucocorticoids cortisol, cortisone and dihydrocortisone and the synthetic glucocorticoids prednisolone, prednisone and methylprednisolone. To this extent, a generic extraction and targeted UHPLC-Orbitrap-HRMS approach, with the possibility of untargeted screening, was developed and validated according to the guidelines of CD 2002/657/EC [26].

2. Material and methods

2.1. Reagents and chemicals

Standards of prednisolone, prednisone, cortisone, cortisol, dihydrocortisone and methylprednisolone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standards were cortisol-d_4 (Sigma-Aldrich) and prednisolone-d_8 (TRC, Canada). Reagents were of analytical grade when used for extraction purposes and obtained from VWR International (Merck, Darmstadt, Germany). The reagents were of LC-MS Optima grade for UHPLC-HRMS application. These were obtained from Fisher Scientific UK (Loughborough, UK). Ultrapure water was obtained by usage of a purified-water system. For filter-sterilization of urine, membrane filters of polyvinylidene fluoride (0.22 µm pore size) were purchased from Millipore (Billerica, USA).

Primary stock solutions were prepared in ethanol at a concentration of 200 µg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at a range of 0.1 – 10 µg mL^{-1}.

2.2. Instrumentation

Analyses were carried out on an UHPLC system, which consisted of an Accela UHPLC pump, an Accela Autosampler and Degasser (Thermo Fisher Scientific, San José, CA, USA). Separation of the glucocorticoids was carried out on a reverse phase Nucleodur C18 Isis UHPLC column (1.8 µm, 100 × 2 mm, Macherey-Nagel, Düren, Germany) at a column oven temperature of 30 °C. The elution gradient was carried out with a binary solvent system consisting of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) at a constant flow rate of 0.3 mL min^{-1}. Optimized separation of all analytes was obtained using a linear gradient starting with a solvent mixture (v/v) of 75% A and 25% B, which was held for 4.0 min. The percentage of acetonitrile was increased to 95% in 0.1 min, and further to 100% in 1.4 min and held there for 2.0 min. Between samples, the column was allowed to re-equilibrate at initial conditions for 1.5 min. A 10 µL aliquot of each sample was injected for analysis. High-resolution mass spectrometric analysis was performed on an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization probe (HESI II), operating in both the positive and negative ionization mode. Ionization source working parameters were optimized and are reported in Table 2.1.

Table 2.1. Instrumental parameters used for HESI (II)-ionization of glucocorticoids.

Instrumental Parameter	Value
Spray voltage	4 kV
Sheath gas flow rate	75 au*
Auxiliary gas flow rate	7 au
Sweep gas flow rate	2 au
Capillary temperature	280 °C
Heater temperature	300 °C
Capillary voltage	45 (-32) V
Tube lens voltage	95 (-100) V
Skimmer voltage	16 (-20) V

*au: arbitrary units

The resolution was set at 50,000 FWHM at 1 Hz and a scan range of *m/z* 150-800 was chosen. The automatic gain control (AGC) target was set at balanced (1 x e^6 ions) and the High Energy Collision Dissociation (HCD) cell was turned off. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific).

2.3. Samples

Urine was collected from five healthy, adult Holstein-Friesian cows, housed at the Institute for Agricultural and Fisheries Research (ILVO) in Merelbeke, Belgium. Urine was collected during spontaneous urination and immediately processed.

2.4. Sample preparation

2.4.1. Fractional factorial design

A sequential strategy of experimental design was used to optimize the analytical extraction of glucocorticoids from urine. The dependent variables that might significantly affect the extraction were screened with a Plackett-Burman design. This design is useful to screen for the main variables from a large number of variables that may affect the response and consists of a two-level design to investigate N-1 variables, with N runs, where N should be a multiple of 4. The variables that might influence the extraction of glucocorticoids in urine were selected based on conditions found in literature [11][23][27][28]. Table 2.2. shows the ten different variables that were investigated as well as the levels of each variable used. The excessive variable was set as dummy [29][30].

Table 2.2. Parameter variable values selected for the Plackett-Burman design.

Variable	Unit	Lower value	Upper value
Volume urine	mL	3	5
pH buffer	-	5	7
Time of hydrolysis	h	1	2
Temperature	°C	37	50
pH adjustment solvent	-	Carbonate buffer	NaOH
Extraction solvent	-	TBME*	Diethylether
Volume of extraction solvent	mL	5	10
Time of shaking	min	5	20
Way of shaking	-	Shaking	Rotation
Volume of 2^{th} LL phase	mL	5	10

*TBME: tert-butyl methylether

In this study, it sufficed to perform 12 experimental runs to specify a particular combination of settings for the different variables included in the design. Next, the main effect of each variable was calculated. When statistical significant differences ($p < 0.05$) were obtained for certain variables, these critical variables i.e. volume of the urine, temperature during hydrolysis and

volume of the extraction solvent were subjected to further optimization by response surface methodology. To this extent, a Central Composite Face (CCF) design was used. The software program Modde 5.0 (Umetrics, Umea, Sweden) was used to carry out the experimental design matrix and data analysis.

2.4.2. Sample extraction

Five mL of blank urine was spiked with the internal standards (cortisol-d_4 and prednisolone-d_8) to obtain a final concentration of 10 µg L^{-1}. A liquid-liquid extraction was performed by adding a volume of 7.5 mL tert-butyl methylether. After 5 min of rotating at 1200 × g, the sample was centrifuged at 5500×*g* for 5 min at 7 °C. The organic layer was collected and transferred to a 15 mL tube. This procedure was repeated by adding 5 mL of tert-butyl methylether to the urine. The organic phase was dried under a gentle stream of nitrogen at a temperature of 50 °C. The residue was redissolved in 100 µL solvent, corresponding to the initial mobile phase conditions and transferred to vials for UHPLC-MS analysis.

2.5. Analytical method validation

Validation of the method was performed by adopting the protocol proposed by Antignac *et al.* (2003) [31]. This protocol was tailored for validating analytical methods based on MS detection and offers a compromise between CD 2002/657/EC [26] and the practical aspects and limitations related to laboratory work. The validation protocol was designed as follows. Analysis of 18 blank pooled bovine urine samples was performed to check the ruggedness of the method and the endogenous level of the natural glucocorticoids cortisol, cortisone and the metabolite dihydrocortisone. This permitted to determine the specificity by calculating the average and standard deviation of the noise amplitude, expressed relative to the selected internal standard signal amplitude. The calibration curves consisted of eight fortification levels. The linearity was evaluated by calculation of the regression coefficient (R^2). The bovine urine samples were fortified with concentrations, ranging from 0.25 to 10 µg L^{-1} for cortisol, cortisone, dihydrocortisone and 1.25 to 50 µg L^{-1} for prednisolone, prednisone and methylprednisolone. For each sample the endogenous concentrations of the natural glucocorticoids, cortisol, cortisone and the metabolite dihydrocortisone, calculated as the average concentration of 18 non-spiked samples, were subtracted from the calculated total concentrations. To evaluate the

precision of the developed analytical method, repeatability and within-laboratory reproducibility were determined and evaluated. Both validation parameters were evaluated by calculating the relative standard deviations (%RSD). For the endogenous compounds, the limit of detection and limit of quantification were determined in 18 unfortified urine samples, as the lowest level at which a compound could be identified with a signal-to-noise ratio greater than 3 and 10 respectively [24].

2.6. Quality assurance

Prior to sample analysis, 10 µL standard mixture of the targeted glucocorticoids at 10 µg L^{-1} was injected to check the operational conditions of the UHPLC-HRMS device. A mixture of the internal standards cortisol-d_4 and prednisolone-d_8, at a concentration of 10 µg L^{-1}, was added to every sample, prior to extraction (Table 2.3.). The compounds were identified based on their retention time relative to the retention time of the internal standard of choice and on the $^{13}C/^{12}C$ isotopic ratio according to the criteria described in CD 2002/657/EC [26]. After identification, the concentrations of the detected glucocorticoids were calculated by fitting their area ratios into eight-point calibrations curves, set up in bovine urine. Area ratios were determined by integration of the area of an analyte under the specific extracted chromatograms in reference to the integrated area of the internal standard.

Table 2.3. UHPLC-HRMS parameters used for the various glucocorticoids with indication of elemental composition, accurate mass, internal standard used, retention time and ionization modus.

Analyte	Elemental composition	Accurate mass (*m/z*)	Internal standard	t_R (min)	Ion mode
Dihydrocortisone	$C_{21}H_{30}O_5$	363.21588	Cortisol-d_4	2.3	+
Cortisol	$C_{21}H_{30}O_5$	363.21588	Cortisol-d_4	3.6	+
Cortisone	$C_{21}H_{28}O_5$	361.20062	Cortisol-d_4	3.9	+
Prednisolone	$C_{21}H_{28}O_5$	361.20062	Prednisolone-d_8	3.4	+
Prednisone	$C_{21}H_{26}O_5$	359.18478	Prednisolone-d_8	3.5	+
Methylprednisolone	$C_{22}H_{30}O_5$	375.21627	Prednisolone-d_8	5.4	+

2.7. Interlaboratory collaborative trial

The Belgian Federal Agency for the Safety of the Food Chain, Laboratories Administration organised a quantitative interlaboratory collaborative trial for prednisolone in urine. The study on the quantitative determination of prednisolone in urine included 5 international laboratories.

Each participant received 18 coded samples (6 samples from slaughterhouses, 4 samples from administration trials and 8 blind duplicates). On these samples, the newly developed and validated UHPLC-HRMS method was applied.

2.8. Stability study

To determine the stability of glucocorticoids in bovine urine, a long-term stability study was set up. The glucocorticoids investigated comprised cortisol, cortisone, dihydrocortisone, prednisolone, prednisone and methylprednisolone. Aliquots of five mL of pooled urine were fortified with a standard mixture of the six glucocorticoids at 20 µg L^{-1}. During the stability study three major parameters were investigated: pH, faecal contamination and redox conditions. In a first batch of samples, the stability of glucocorticoids was tested under different pHs. The urine aliquots were brought to pH 1, 3, 5, 7 or 10 by means of HCl or NaOH. During a second batch, urine samples were divided into three groups. The first group comprised urine with 0.5% of faecal contamination. The second group consisted of filter-sterilized urine (0.22 µm pore size) and the last group comprised of untreated urine. In the last part of the study, urine samples with or without faecal contamination were flushed with N_2 during 30 min to obtain an anaerobe environment (Figure 2.1.).

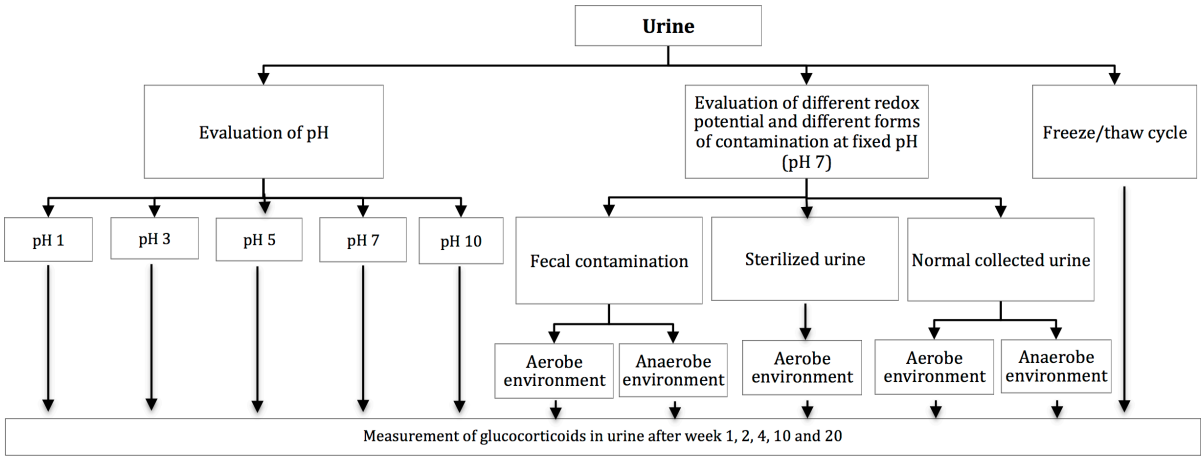

Figure 2.1. Overview of the experimental protocol of the stability study.

For each time point in the stability study and for each parameter set, samples was prepared in triplicate and stored respectively at -80 °C, -20 °C, 4 °C or at room temperature (15 - 20 °C) up to 20 weeks. After week 1, 2, 4, 10 and 20, the six glucocorticoids in each batch of samples were quantitatively analysed. Additionally, the effect of freeze/thaw cycles was investigated by

consecutively thawing the frozen samples (i.e. remove from freezer and allow to thaw at room temperature) in the first week, refreezing the samples and finally thawing them again for analysis at the next time of sampling (after 2, 4, 10 and 20 weeks).

2.9. Statistical analysis

To evaluate the Plackett-Burman design, a one-way analysis of variance (ANOVA) test was performed using Modde 5.0. Data of the stability study were analysed by multiple linear regression analysis using SPSS Statistics 20 (IBM, United States). A p-value below 0.05 indicated a significant difference.

3. Results and discussion

3.1. Optimization of extraction procedure

A Plackett–Burman experimental design was employed to statistically evaluate the effects of ten variables in the extraction procedure [17][29][30]. The following variables were considered: volume of the urine, pH of the buffer during hydrolysis, time of hydrolysis, temperature during hydrolysis, pH adjustment solvent, type of extraction solvent, volume of extraction solvent, duration of shaking, way of shaking, volume of the second liquid-liquid extraction phase (Table 2.2.). To identify the most significant variables, the difference between a low and high level was set in this way to obtain a maximal statistical difference. This experimental design was executed in a random order (Supplementary table).

The volume of the urine, the temperature during hydrolysis and the volume of the second liquid-liquid extraction phase, demonstrated significant positive effects on the extraction efficiency ($p < 0.05$). It was observed that for methylprednisolone and dihydrocortisone, the extraction solvent tert-butyl methylether had a positive regression coefficient, which resulted in an increased recovery. The same effect was noticed for NaOH as pH adjustment solvent and rotation as way of shaking (Supplementary table). After further optimization of the main variables with a CCF design (Figure 2.2.), the most promising settings were included into the extraction procedure.

The absolute areas of the individual compounds were considered to evaluate the absolute effects of the different variables. Finally, an extraction procedure without hydrolysis was selected in order to maintain the generic character of the extraction and to ensure the potential of a full-scan untargeted screening in a later phase (cfr. 2.4.2).

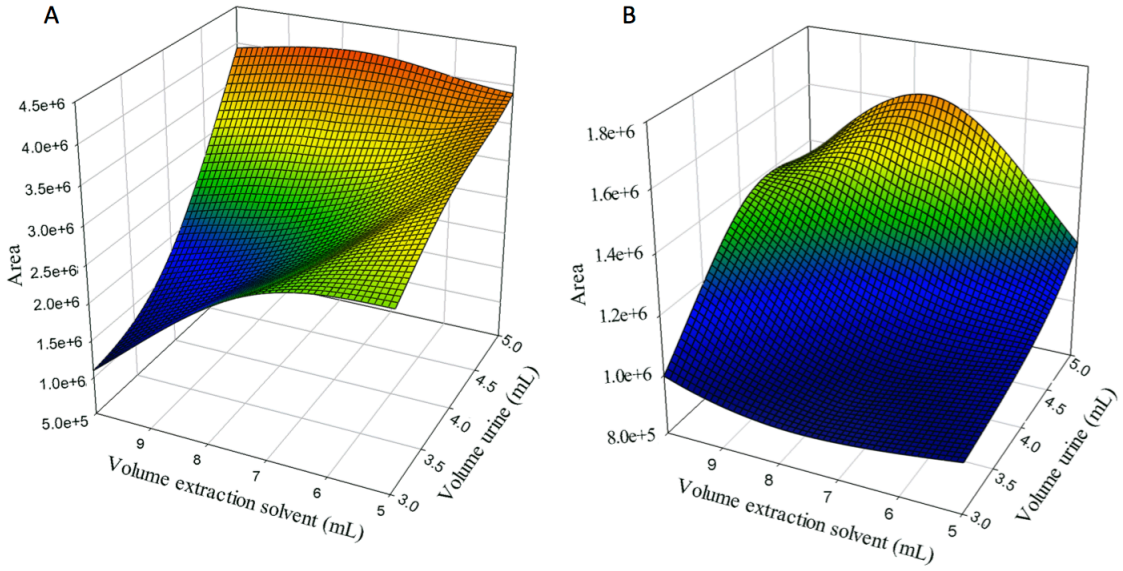

Figure 2.2. Response surface plots obtained for the variables volume of the extraction solvent and volume of the urine for cortisol (A) and prednisolone (B) after statistical analysis of the Plackett-Burman experimental design.

3.2. UHPLC and MS parameters

Due to their equal molecular masses and similar chromatographic behavior, the separation of prednisolone and cortisone poses a true analytical challenge. For the chromatographic separation, two different columns were tested, i.e. the Nucleodur C18 Isis (1.8 µm, 100 × 2 mm, Macherey-Nagel) and Acquity BEH C18 (1.7 µm, 100 × 2.1 mm, Waters) columns. Based on the achieved baseline peak separation of prednisolone and cortisone and the retention time of the first and last eluting analytes, the Nucleodur C18 Isis column was retained. For additional separation of prednisolone and cortisone several flow rates (from 0.3 to 0.5 ml min^{-1}) and different column oven temperature settings (from 20 to 50 °C) were tested. Several mobile phases were tested including 0.1% and 0.2% formic acid in acetonitrile/water; 0.1% and 0.2% acetic acid in acetonitrile/water, where the optimal mobile phases solvents were 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, as Touber *et al.* (2007) demonstrated

before [11]. The optimal retention times were achieved by careful adaption of the gradient program.

Before determining the optimal MS conditions, each glucocorticoid standard and deuterium-labelled internal standard (10 µg L^{-1}) was infused on the HRMS and the observed masses were compared with the theoretical masses, which were calculated using Xcalibur 2.1 software. The mass deviations, expressed in parts per million (ppm) and defined as: 10^6 x [(measured mass – theoretical mass) / theoretical mass], were found to be below 2 ppm. The CD 2002/657/EC states, in case of HRMS application, that the presence of the $[M+H]^+$ or $[M-H]^-$ ion with their specified retention times and respective accurate masses is insufficient for the identification and confirmation of the selected analytes, since at least two diagnostic ions are requisite [26]. Therefore, the mono-isotopic pattern (^{13}C ion) may assist in the further confirmation of a compound's identity. An isotopic ion was only found suitable as a diagnostic ion when the corresponding $[M+H]^+$ or $[M-H]^-$ was also detected and the calculated relative ion intensity was in compliance with CD 2002/657/EC requirements [26].

Instrumental HRMS parameters, which are reported in Table 2.1., were optimized based on the peak intensities, peak areas and signal-to-noise ratios of the individual analytes. The optimal AGC value was found to be balanced scan (1 x e^6 ions). Based on the analysis of fortified urine samples at different mass resolutions (i.e. 50,000 and 100,000 FWHM) a mass resolution of 50,000 FWHM at 1 Hz was selected.

3.3. Method validation

During the validation pooled urine was used. This was a mixture of visually different types of urine (dark and light coloured). Dark urines indicate a strong abundancy of matrix interferences. As a consequence, a good outcome of the validation using dark urine allows expecting equal or even better results when using other urine samples. The analytical method was validated for quantitative confirmation [26]. The performance characteristics of the validation are presented in Table 2.4.

Table 2.4. Decision limits (CC_α) and detection capabilities (CC_β) calculated for prednisolone, prednisone and methylprednisolone in urine according to 2002/657/EC [26]. Limits of Detection (LOD) and Limits of Quantification (LOQ) calculated for cortisol, cortisone and dihydrocortisone in urine. Overview of main performance characteristics for the six glucocorticoids, analysed in urine, according to 2002/657/EC [26].

Analyte	LOD[1] CC_α[2] (µg L^{-1})	LOQ[1] CC_β[2] (µg L^{-1})	Nominal concentration (µg L^{-1})	Recovery (%)	Precision	
					Repeatability RSD (%)	Intra-lab. reprod. RSD (%)
Dihydrocortisone	0.19[1]	0.62[1]	0.5	90	9.21	14.38
			1	95	9.34	9.44
			2	100	5.82	8.58
Cortisol	0.25[1]	0.83[1]	0.5	95	6.38	13.07
			1	100	5.63	7.96
			2	100	3.32	5.24
Cortisone	0.10[1]	0.30[1]	0.5	106	13.97	16.45
			1	103	9.50	12.89
			2	104	9.65	11.34
Prednisolone	0.09[2]	0.37[2]	2.5	97	7.78	6.78
			5	97	2.49	3.22
			10	94	2.88	3.47
Prednisone	0.10[2]	0.29[2]	2.5	90	5.36	10.59
			5	95	4.22	4.15
			10	95	4.74	4.95
Methylprednisolone	0.50[2]	0.79[2]	2.5	85	6.79	13.41
			5	91	5.91	7.48
			10	90	5.08	9.89

1: natural glucocorticoids, 2: synthetic glucocorticoids

3.3.1. Specificity

Since the 'blank' samples, i.e. samples that were not enriched with glucocorticoids, contained cortisol, cortisone and the metabolite dihydrocortisone [32], an actual blank sample was not available. As a consequence, specificity could not be evaluated for all targeted glucocorticoids by comparing the chromatograms of blank and enriched samples. For the natural glucocorticoids, cortisol and cortisone and the metabolite dihydrocortisone, specificity was exclusively based on the chromatograms from 18 non-fortified urine samples. These chromatograms indicated that no other matrix substances significantly interfered with these glucocorticoids since signal-to-noise ratios were at least 3. To evaluate the specificity of the other glucocorticoids, 18 urine samples were fortified with glucocorticoids standards to reach concentrations of 5 µg L^{-1} for prednisolone, prednisone and methylprednisolone. For each analyte spiked, the obtained chromatograms showed a significant increase in peak area at the specific retention time of the

compounds, taking a signal-to-noise ratio of at least 3 into account (Figure 2.3.). As a result, the developed method was found to be specific for cortisol, cortisone, prednisolone, prednisone, dihydrocortisone and methylprednisolone in the presence of matrix compounds.

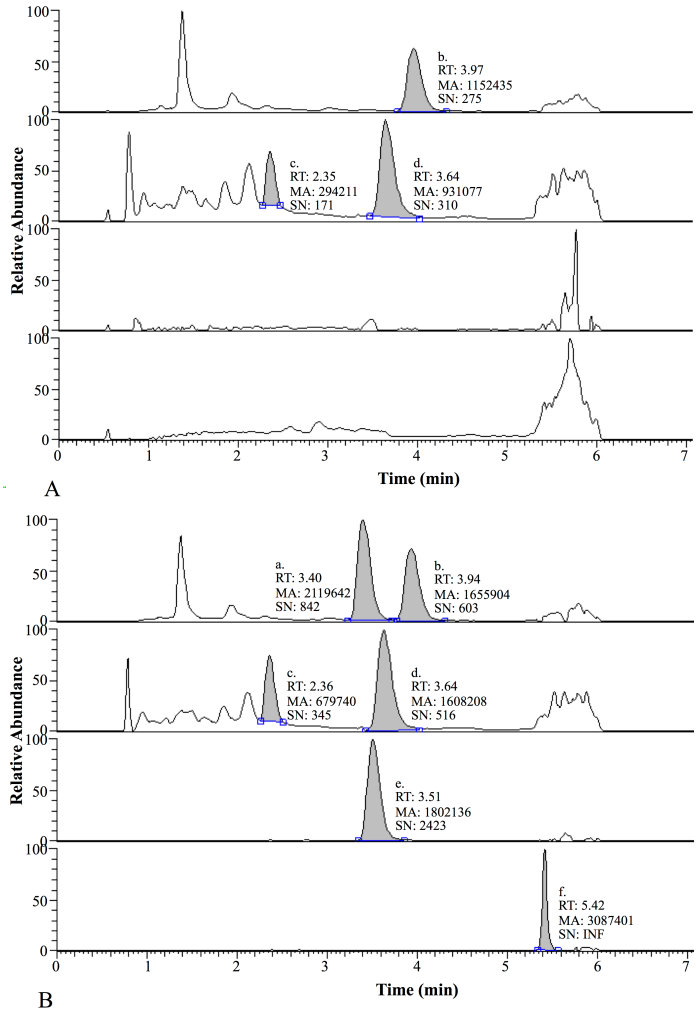

Figure 2.3. Chromatogram of a 'blank' urine sample, i.e. a sample that was not enriched with glucocorticoid standards (A), and chromatogram of a urine sample fortified with a. Prednisolone 5 µg L^{-1}; b. Cortisone 1 µg L^{-1}; c. Dihydrocortisone 1 µg L^{-1}; d. Cortisol 1 µg L^{-1} ; e. Prednisone 5 µg L^{-1} ; f. Methylprednisolone 5 µg L^{-1} (B). The maximum mass deviation was set at 2 ppm.

3.3.2. Selectivity

Analytes were identified on the basis of their relative retention time, i.e. the ratio of the retention time of the analyte to that of the corresponding internal standard [26]. In addition, the accurate mass of the ions ($[M+H]^+$) was taken into account when the chromatographic peak of interest had a signal-to-noise of at least 3. A maximum mass deviation of 2 ppm was allowed within this study.

3.3.3. Linearity

Eight-point calibration curves in 'blank' samples were used to evaluate the linearity of the developed method for the different compounds. The samples were fortified with concentrations, ranging from 0.25 to 10 µg L^{-1} for cortisol, cortisone, dihydrocortisone and 1.25 to 50 µg L^{-1} for prednisolone, prednisone and methylprednisolone. For each sample the endogenous concentration, which was calculated as the average concentration of 18 non-spiked samples, was subtracted from the calculated total concentration. Linearity performed well since regression coefficients (R^2) were all ≥ 0.99.

3.3.4. Precision

To evaluate the repeatability, three series of six replicates of urine samples were analysed, and this at three fortification levels. For the natural glucocorticoids, cortisol, cortisone and the metabolite dihydrocortisone, the fortification levels were based on the determined endogenous concentrations and are reported in Table 2.4. For the synthetic glucocorticoids, prednisolone, prednisone and methylprednisolone, the fortification levels were based on the established maximum residue limits (4 µg kg^{-1} and 10 µg kg^{-1} in bovine muscle for prednisolone and methylprednisolone respectively) [33],[34]. These analyses were carried out on different occasions by the same operator under repeatable conditions. To evaluate the repeatability for the three fortification levels as such, additional calculations were made for the natural glucocorticoids and the metabolite, dihydrocortisone, whereby for each sample the endogenous concentration was substracted from the calculated total concentration. The endogenous concentration was calculated as the average concentration of 18 'blank' samples. As presented in Table 2.4., the calculated RSD values were below 10% for the targeted glucocorticoids, which indicated excellent repeatability since values have to be below 15% according to CD 2002/657/EC [26].

The within-laboratory reproducibility was evaluated with four series of six replicates of samples, at three fortification levels. These analyses were carried out on different days, by different operators. The calculated RSD values for all glucocorticoids were below 15%, as presented in Table 2.4. This indicates a very good within-laboratory reproducibility, following CD 2002/657/EC [26].

3.3.5. Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were defined as the lowest level at which a compound could be identified in blank urine samples with a signal-to-noise ratio greater than 3 and 10 respectively [24]. For the natural glucocorticoids, cortisol, cortisone and the metabolite dihydrocortisone, the limit of detection and limit of quantification were theoretically calculated based on eight-point calibration curves in matrix. The LOD ranged from 0.10 to 0.25 μg L^{-1} for cortisol, cortisone, dihydrocortisone and the LOQ ranged from 0.30 to 0.83 μg L^{-1} (Table 2.4.). Compared to other studies, the developed method resulted in comparable or better LOD and LOQ values [6][9][10][11].

3.3.6. Decision limit (CC_α) and detection capacity (CC_β)

For the synthetic glucocorticoids, prednisolone, prednisone and methylprednisolone, decision limits (CC_α) and detection capabilities (CC_β) were calculated according to the guidelines of CD 2002/657/EC [26]. The CC_α was calculated as the mean of the noise amplitude plus 2.33 times the standard deviation of the noise amplitude. For calculating CC_β, 18 blank urine samples were spiked at the determined CC_α level, subsequently the CC_β was determined by calculating the mean concentration at the corresponding decision limit plus 1.64 times the standard deviation of the mean measured concentration [26]. Table 2.4. summarizes the calculated CC_α and CC_β values for the different glucocorticoids. Decision limits and detection capabilities ranged respectively, from 0.09 to 0.50 μg L^{-1} and from 0.29 to 0.79 μg L^{-1}.

3.3.7. Mean recovery

As no certified reference material was available, trueness was determined as the mean corrected recovery by using fortified urine samples. To this end, three fortification levels were considered with six replicates for each level. For each sample, calculated concentrations were adjusted with the determined endogenous concentration levels where necessary. The calculated mean recoveries ranged from 85 to 106% and fullfilled the CD 2002/657/EC criteria (Table 2.4.) [26].

3.3.8. Interlaboratory collaborative trial

The results of the ring trial demonstrated that all methods used, were suited for the detection and identification of prednisolone in urine. The measurement uncertainty (%) and the bias (%) of the methods ranged respectively from 42.9 to 54.4 % and -16.7 to 18.5 %. Our newly developed method ranked second in performance with a measurement uncertainty of 44.8% and a bias of -10%, which may be considered as satisfactory taking into account the low concentrations of prednisolone that were present in the test samples (0.38 - 5.49 µg L^{-1}).

3.4. Stability of glucocorticoids in urine

In recent years, the high frequency of bovine urine samples found positive for prednisolone has raised many questions. One hypothesis put forward is the potential transformation of the natural glucocorticoids cortisol and cortisone into prednisolone and prednisone, respectively [9][10]. The concentration of natural steroids present in urine may alter by inappropriate storage and the presence of microorganisms [22][23]. For this reason the influence of different conditions of sample handling and storage were examined. Studied storage conditions included temperature (room temperature, refrigerator, and freezer), storage time, sample pH and faecal contamination. The Variable Importance on Projections plot shows that the contributions of temperature during conservations had the most impact, followed by pH and time (Figure 2.4.).

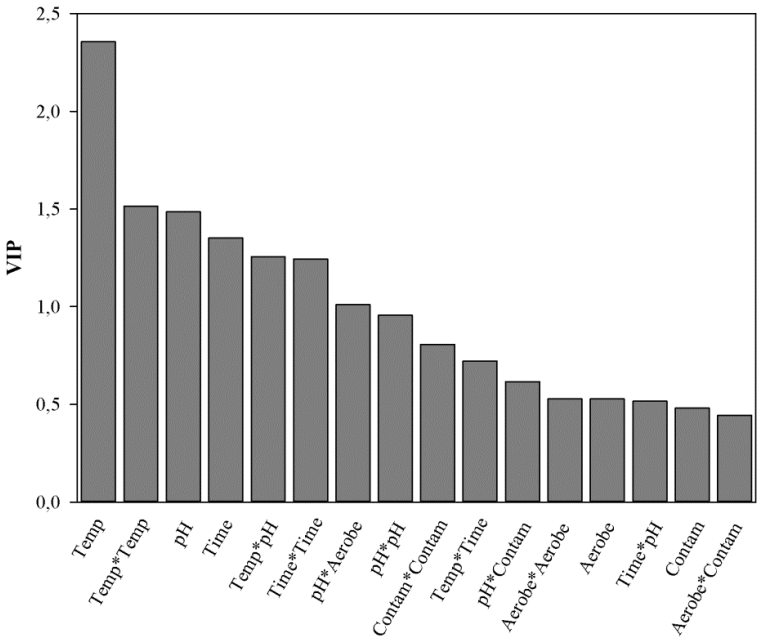

Figure 2.4. Variable Importance on Projection plot with Temp: temperature and Contam: faecal contamination.

3.4.1. Effect of pH at different temperatures

It was noticed that the observed concentrations of glucocorticoids were highly dependent on the temperature of preservation and the pH-value. In the present study, changes in glucocorticoid concentrations in bovine urine samples stored at various pH-values for up to 20 weeks were examined (Figure 2.5.).

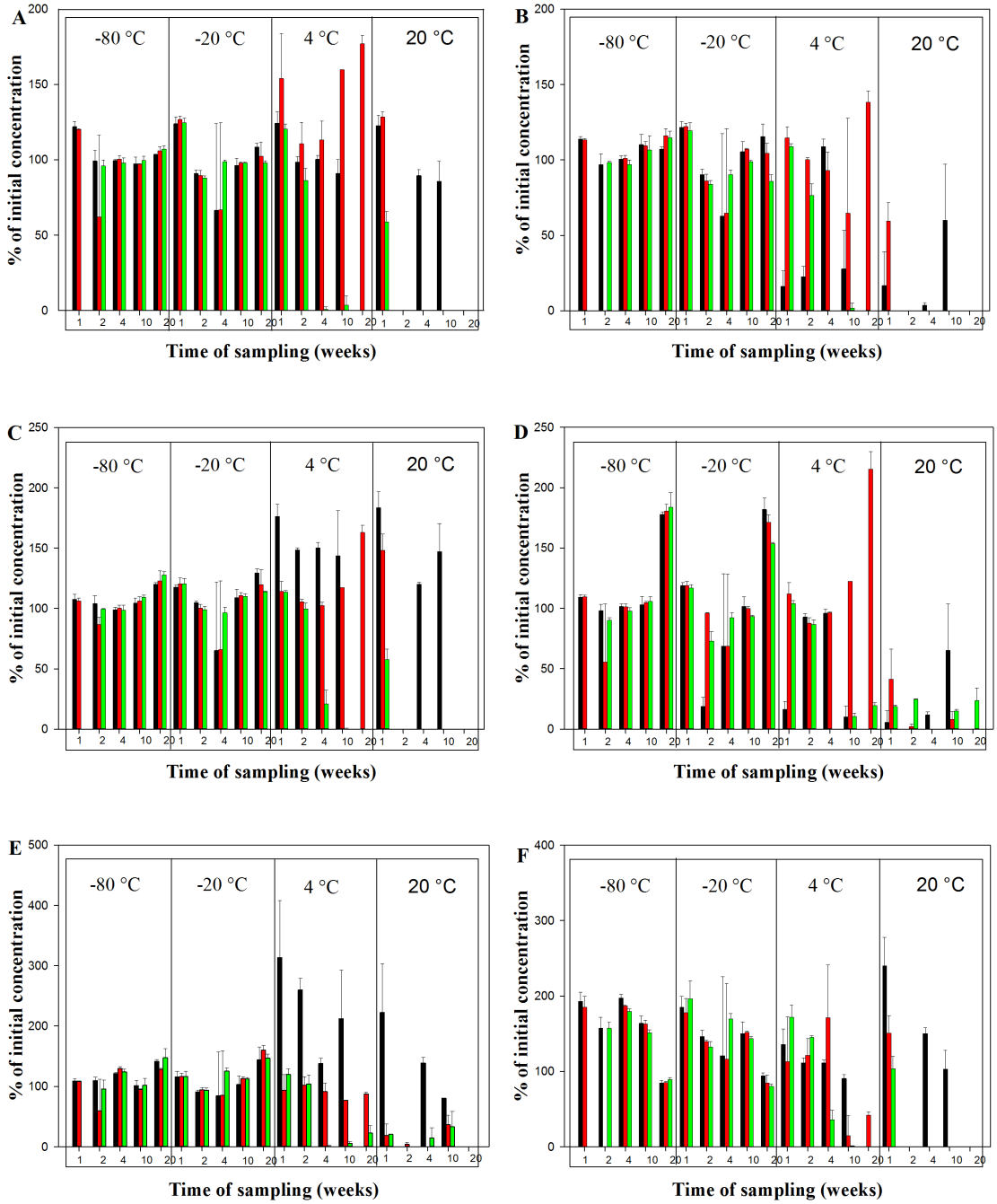

Figure 2.5. Concentration of the different glucocorticoids in urine at different pH-values and storage temperatures (A) Cortisol; (B) Cortisone: (C) Prednisolone; (D) Prednisone; (E) Dihydrocortisone; (F) Methylprednisolone with pH 1 (Black); pH 5 (Red); pH 10 (Green).

For all glucocorticoids, a significant difference ($p < 0.05$) was noticeable between the different pH values of the samples (pH 1, pH 3, pH 5, pH 7 or pH 10) when preserved at 4 °C and room temperature. In figure 2.5. the results at pH 1, pH 5 and pH 10 are presented. Under acidic conditions, the highest concentrations were retrieved for all analytes while a pH-value higher than 3 significantly stimulated the degradation of glucocorticoids in urine. In terms of sample storage this implies that a pH-value lower than 3 allows preservation for up to 10 weeks. At extreme pH-values the denaturation of microbial (e.g. *Escherichia coli)* enzymes may occur. This might cause various interruptions to the biochemical processes and kill of bacteria, thereby preventing microbial degradation of glucocorticoids. For many microorganisms a pH of 5 to 7 is, however, optimal for enzymatic and bacterial activity [6][35], which can be confirmed by the observation that at pH-values higher than 3, significant degradation of glucocorticoids in urine occurred. This implies that glucocorticoid recoveries negatively correlate with urine pH. On the other hand, when stored at -20 °C and -80 °C, glucocorticoids displayed an analogous behaviour, irrespective of the pH of the urine.

3.4.2. Effect of faecal contamination at different temperatures

Based on literature findings faecal contamination of urine may play a role in the transformation of cortisol into prednisolone [25]. Hence, faecal contamination was included as a test condition in this study. Major discrepancies are, however, noticed between the different contaminated samples depending on the temperature of preservation. When urine samples are stored in the freezer (-20 °C and -80 °C), glucocorticoids displayed an analogous behaviour irrespective of the contamination status (Figure 2.6.). The glucocorticoid concentrations in urine samples stored at 4 °C and at room temperature encountered more variation throughout time. After 1 week of preservation, at different temperatures, no significant differences in concentrations could be observed between urine samples with and without faecal contamination and filter-sterilized urine. After 2 weeks, the concentration in faecally contaminated urine dropped ca. 20% for the six glucocorticoids, while for the non-faecally contaminated urine a gradual decrease could be noticed.

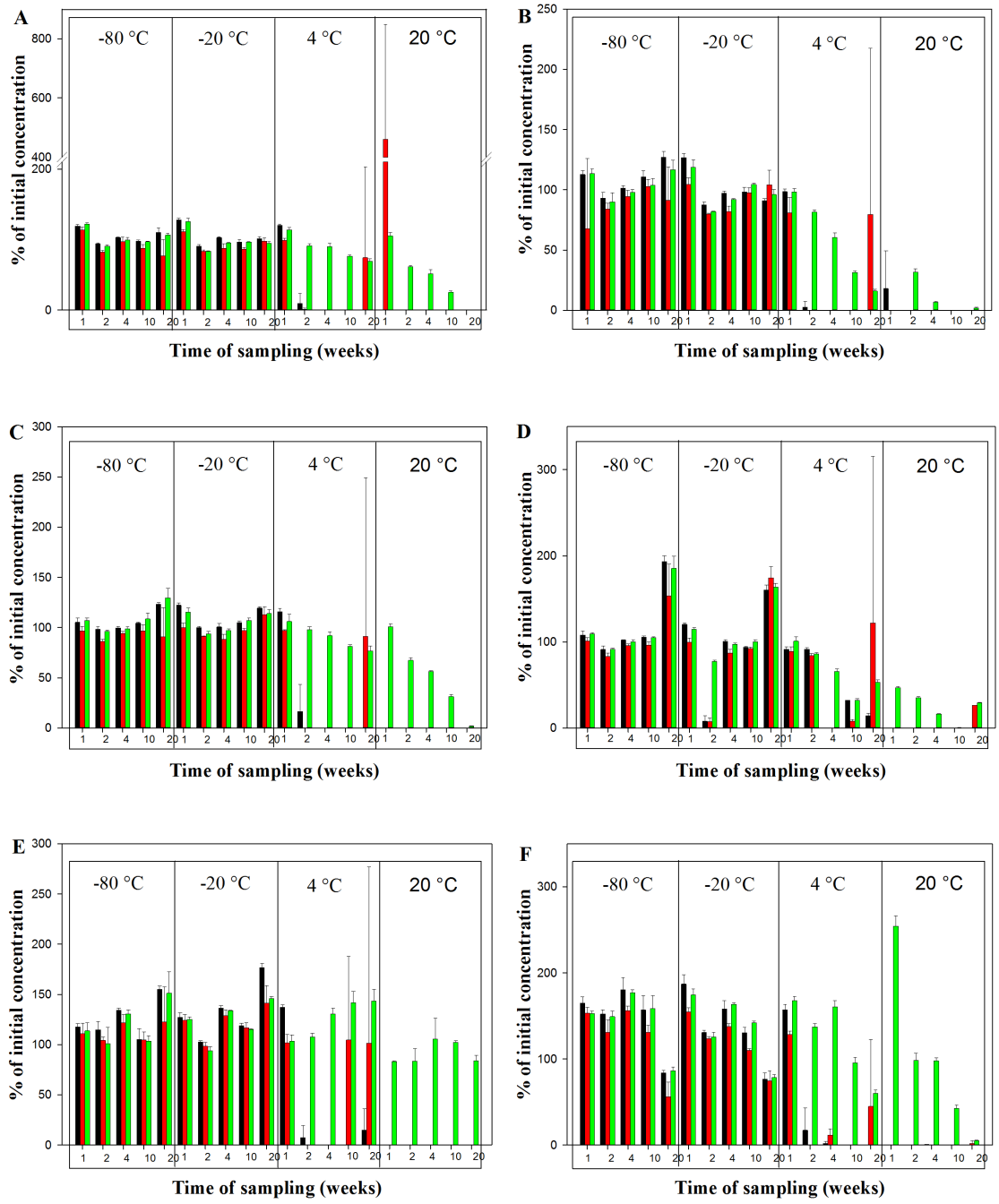

Figure 2.6. Concentration of the different glucocorticoids in urine at different contamination degrees and redox conditions (A) Cortisol; (B) Cortisone; (C) Prednisolone, (D) Prednisone; (E) Dihydrocortisone; (F) Methylprednisolone with Faecal contamination O_2 (Black); Faecal contamination N_2 (Red); Sterile urine (Green).

It is known that bacterial growth is well sustained in urine, especially, when specimens are not frozen immediately upon sampling [23]. This may explain the observed degradation in most of the samples. On the other hand, also the increase in concentration after 20 weeks of preservation in urine at -20 °C and -80 °C could be explained by induced bacterial hydrolysis of

conjugates through several microbial enzymes (e.g. *Escherichia coli*, *Nocardioides simplex*, *Aspergillus flavus*) [22][25][36][37]. Only in one urine sample, however, an increased concentration of prednisolone was found after faecal contamination under an anaerobic environment when preserved at 4 °C during 20 weeks. This was the only case of neoformation of prednisolone. Faecal contamination under aerobe conditions did, however, not promote the neoformation of prednisolone or prednisone in contrast to the results of Arioli *et al.* (2010) [25].

Only in filter-sterilized urine, five glucocorticoids appeared up to 20 weeks at room temperature. Jiménez *et al.* (2006) demonstrated that differences between testosteron and epitestosteron concentrations obtained before and after filtration of urine were not statistically significant ($p < 0.05$) [38]. This indicates that filtration of urine has no influence on the initial concentration. It may as a result be concluded that prior to long-term preservation of steroidal substances such as glucocorticoids, filtration (over 0.22 µm filters) is recommended.

3.4.3. Freeze-thaw cycles

Evaluation of freeze and thaw stability was performed by preservation of glucocorticoids in urine at -20 °C. Each sample was subjected to five freeze-thaw cycles at each round of sampling during the long-term stability study. All glucocorticoids showed a similar progress over time except for methylprednisolone, for which a significant decrease in concentration could be noticed only after five freeze-thaw cycles (Figure 2.7.).

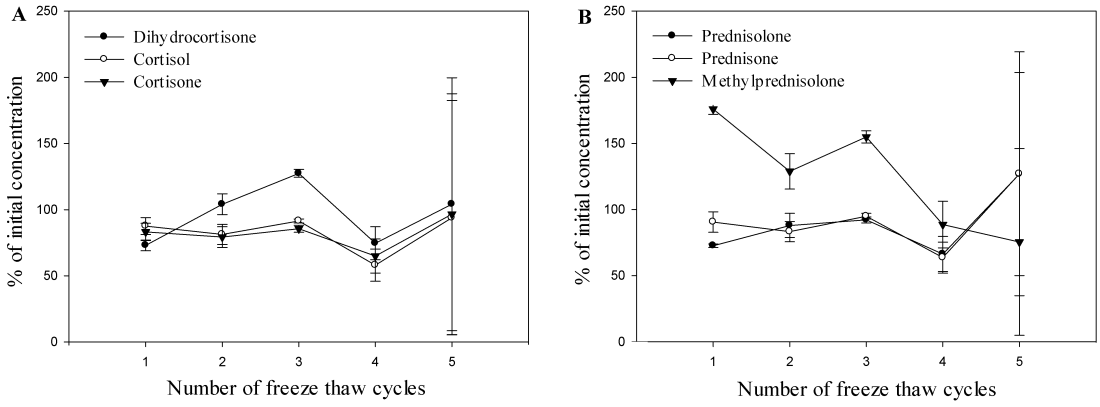

Figure 2.7. Concentration of the different glucocorticoids in urine exposed to a number of freeze-thaw cycles. (A) Natural glucocorticoids; (B) Synthetic glucocorticoid.

4. Conclusion

In this study the successful development and validation of a generic extraction procedure and detection method for glucocorticoids in urine according to the criteria set in Commission Decision 2002/657/EC was described [26]. The first step within the development of this generic extraction protocol comprised the identification of the variables that significantly affect the recoveries of the natural glucocorticoids cortisol, cortisone and the metabolite dihydrocortisone, and the synthetic analogues prednisolone, prednisone and methylprednisolone in urine. Therefore a Plackett-Burman experimental design was set up. This design allowed to develop, in the most efficient statistically proven manner, a generic extraction procedure and reduced the number of experiments. Besides, the suitability of the UHPLC-Orbitrap-HRMS platform for the targeted detection of the free glucocorticoids, i.e. cortisol, cortisone, prednisolone, prednisone and methylprednisolone and the metabolite dihydrocortisone was demonstrated. Since this method employs a full scan working principle, it allows retrospective screening, i.e. screening for non a-prior selected analytes and unidentified, unknown compounds and metabolomics studies. The long-term stability study (20 weeks) executed in this study provided evidence for the optimal conditions for medium to long-term storage of glucocorticoids in bovine urine. To preserve glucocorticoids in bovine urine for a long period (20 weeks) it is recommended to filter-sterilize the urine and preserve under acidic conditions, preferentially at pH 3 and at a temperature of -80 °C (or at least -20 °C).

Supplementary table 1. The coefficients and p-values of the compounds in relation with the variables after implementation of a Plackett-Burman experimental design.

	Cortisol		**Cortisone**		**Dihydrocortisone**		**Prednisolone**		**Prednisone**		**Methylprednisolone**	
	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value
Volume urine	491054	0.0025*	220968	0.0002*	154116	0.0811	311622	0.0017*	294468	0.0033*	238456	0.0004*
pH buffer	42615	0.5895	5800.06	0.7540	121239	0.1420	16142.1	0.7178	15757	0.7542	-5661.72	0.8135
Time of hydrolysis	111944	0.1987	26063.1	0.2060	-62144.7	0.4025	71756.8	0.1597	64643.3	0.2409	63686.8	0.0471*
Temperature	-267947	0.0211*	-100745	0.0043*	58802.3	0.4261	-150495	0.0224*	-170709	0.0220*	-7367.53	0.7594
pH adjustment solvent												
Carbonate buffer	-46488.4	0.5667	-45205	0.0631	-138468	0.1117	-23387	0.6126	-30221	0.5642	12451.3	0.6174
NaOH	46488.4	0.5667	45205	0.0631	138468	0.1117	23387	0.6126	30221	0.5642	-12451.3	0.6174
Extraction solvent												
TBME	119397	0.1845	1799.6	0.9239	165073	0.0723	70440.3	0.1739	42862.9	0.4236	108967	0.0090*
Diethylether	-119397	0.1845	-1799.6	0.9239	-165073	0.0723	-70440.3	0.1739	-42862.9	0.4236	-108967	0.0090*
Volume of extraction solvent	246890	0.0274*	63015.8	0.0218*	235005	0.0240*	113285	0.0428*	132081	0.0482*	-47948.5	0.0998
Time of shaking	30860.4	0.6933	12615.4	0.5059	-104600	0.1904	26219.7	0.5629	24451.3	0.6302	30709.5	0.2435
Way of shaking												
Rotation	7279.43	0.9269	-12446	0.5209	78289.6	0.3142	7509.18	0.8687	-8817.8	0.8635	53029.9	0.0826
Shaking	-7279.43	0.9269	12446	0.5209	-78289.6	0.3142	-7509.18	0.8687	8817.8	0.8635	-53029.9	0.0826
Volume of 2th LL phase	-100814	0.2381	-29006.6	0.1686	-130420	0.1211	-53373.6	0.2689	-50057.4	0.3467	-25569.6	0.3187

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CHAPTER III

DEVELOPMENT AND VALIDATION OF A HIGH-RESOLUTION MASS SPECTROMETRY BASED METHOD TO STUDY THE LONG-TERM STABILITY OF NATURAL AND SYNTHETIC GLUCOCORTICOIDS IN FAECES

Adapted from:

De Clercq, N., Vanden Bussche, J., Croubels, S., Delahaut, P. and Vanhaecke, L. (2014) Journal of Chromatography A. 1336, 76-86.

Abstract

Faecal glucocorticoid analysis is a powerful non-invasive tool for the study of the animal endocrine status and stress physiology, which is mainly carried out by immunoassays, characterized by some limitations. In this study, an ultra high-performance liquid chromatography coupled to high-resolution Orbitrap mass spectrometry (UHPLC-HRMS) method was developed to confirm the presence of glucocorticoids in bovine faeces during a long-term stability study. Because of the complex nature of faeces, an appropriate extraction and purification procedure was developed. To this extent, a Plackett-Burman experimental design was successfully applied to determine the key conditions for optimal extraction of glucocorticoids from faeces. The targeted analysis, including natural and synthetic glucocorticoids, was successfully validated according to CD 2002/657/EC. Decision limits and detection capabilities for prednisolone, prednisone, methylprednisolone and the metabolites 20α-dihydroprednisolone and 20β-dihydroprednisolone ranged, respectively, from 0.15 to 2.95 μg kg^{-1} and from 0.40 to 5.20 μg kg^{-1}. For the natural glucocorticoids limits of detection and limits of quantification for dihydrocortisone, cortisol and cortisone ranged, respectively, from 0.55 to 2.10 μg kg^{-1} and from 0.70 to 5.00 μg kg^{-1}.

The stability study of glucocorticoids in faecal matrix demonstrated that lyophilizing the faeces, storage at -80 °C, and aerobe conditions were optimal for preservation and able to significantly ($p < 0.05$) limit degradation up to 10 weeks.

1. Introduction

The glucocorticoids cortisol and cortisone are steroid hormones naturally synthesized in the adrenal cortex. Their well-known anti-inflammatory properties have led to the development of synthetic glucocorticoid analogues, which exert even higher anti-inflammatory activities i.e. betamethasone, dexamethasone, methylprednisolone and prednisolone, with prednisone as prodrug [1]. In the European Union, these compounds are permitted for therapeutic use in livestock. Beside the anti-inflammatory properties, these drugs also induce body weight gain in production animals by improving feed intake and lowering feed conversion. Due to their growth-promoting effects and the potential consumer's health risks of residues thereof [2][3], the use of synthetic glucocorticoids in livestock has been strictly regulated in the European Union [4], by setting maximum residue limits for betamethasone, dexamethasone, methylprednisolone and prednisolone in selected tissues of animal origin [5].

In the frame of the National Residue Monitoring Plans, liver, urine and faeces are frequently analysed to ensure the absence of residues in food products of animal origin and to detect possible illegal use as growth-promoter [6][7]. The European Union Reference Laboratories made a consensus to set the MRPL for prednisolone in bovine urine at 5 μg L^{-1} [8]. While liver samples can be merely obtained upon slaughtering [9][10] urine and faeces are easily accessible through non-invasive sampling. The use of faecal samples has some advantages over that of urine when focusing on the hormonal status of the animals and their long-term endocrine profile [11][12]. The sampling is easy, does not interfere with the stress response itself and permits on-farm monitoring. Faecal analyses are increasingly being used to examine glucocorticoids as a potential indicator of adrenal activity and animal stress [13]. They reflect an average level of circulating glucocorticoids over a time period, rather than a point sample, since the levels in faeces are less affected by episodic fluctuations or the pulsatility of hormone secretion. Therefore the measured faecal glucocorticoid concentrations might represent the hormonal status of an animal more accurately than in a single plasma or urine sample [13][14][15][16].

In recent years, a higher frequency of prednisolone positive bovine urines has been observed [17][18]. Several hypotheses have been put forward for this finding including the influence of stress evoked by handling before slaughter and the resulting conversion from cortisol and

cortisone to respectively prednisolone and prednisone [19][20]. This has rendered the analysis of glucocorticoids into a complicated business, since besides the mere presence of residues, their origin (either endogenous and/or exogenous) has become a matter of debate. As the glucocorticoid metabolism gives rise to large number of derivates with similar chemical structures and molecular weights, the search for biomarker candidates proved quite challenging [21][22]. At this point, one of the principal metabolites of prednisolone, i.e. 20β-dihydroprednisolone has been put forward as potential biomarker for specifying endogenous traces of prednisolone [22][23].

In the past, steroid analysis in faecal samples was mainly carried out by immunoassays [14][24]. Although these techniques have proven their usefulness in wildlife studies [25][26], some limitations exist with respect to specificity. Cross-reactivity of the specific antibody with other similar steroids can lead to controversial results [27][28]. In this context, LC-MS techniques are more fit to distinguish similar glucocorticoid compounds [29]. Ultra-high performance liquid chromatography (UHPLC) using columns with sub 2 µm particles, which results in a higher chromatographic resolution, are commonly used these days [30]. From literature, it may be concluded that tandem MS using selected reaction monitoring is currently the preferred detection method for glucocorticoid analysis [12][29]. An inherent limitation of this targeted approach is the inability to screen for unidentified and unknown compounds such as metabolites. Therefore in this study, the UHPLC system was coupled to a high resolution Orbitrap mass spectrometer, which allows the production of full scan MS spectra with a resolving power up to 100,000 FWHM and a high mass accuracy (mass deviations below 2 ppm) [31]. This detection technique offers the possibility to simultaneously analyze a virtually unlimited number of compounds, provides sufficient selectivity for complex matrix extracts such as faeces and allows post-acquisition re-interrogation of data and screening for unidentified and/or unknown compounds.

Because of the complex nature of faeces, appropriate sample preparation procedures are required, but in terms of the metabolomic approach to be kept as generic as possible. To this extent, Plackett-Burman experimental design is a useful tool to screen for the main variables within a large number of variables that may affect the extraction yield [32]. This highly efficient

design provides the opportunity to identify the significant extraction conditions with a minimum of experiments.

Although the use of non-invasive sampling techniques has increased, several confounding factors inhibit its wide spread use [33]. A long-term stability study of glucocorticoids in urine has for example shown that the environmental conditions during preservation have a big influence on the recovery [34]. This is particularly true for urine contaminated with faecal material which can contain a microbial flora up to 10^{11} CFU/g faeces [35]. This microbial activity may seriously interfere with the concentration of the extracted compounds as it has been shown that bacteria and bacterial enzymes in faeces decompose steroid metabolites within hours in untreated faeces [24][36]. Therefore, in the present study, an extensive stability study of glucocorticoids in bovine faeces was performed in which the effect of different storage conditions such as lyophilization and temperature were considered. Additionally, the preservations under aerobe and anaerobe environments, as well as the addition of ethanol was evaluated. Furthermore, this stability study included the determination of losses during multiple freeze-thaw cycles. The compounds of interest were the natural glucocorticoids cortisol, cortisone and dihydrocortisone (4-pregnene-17α,20β,21-triol-3,11-dione) and the synthetic glucocorticoids prednisolone, prednisone and methylprednisolone and several potential biomarker candidate prednisolone metabolites including 20α-dihydroprednisolone and 20β-dihydroprednisolone (Figure 3.1.). To this extent, a generic extraction and analytical method to measure glucocorticoids and a number of their metabolites in faecal samples of cattle was developed and validated according to the guidelines of CD 2002/657/EC [37].

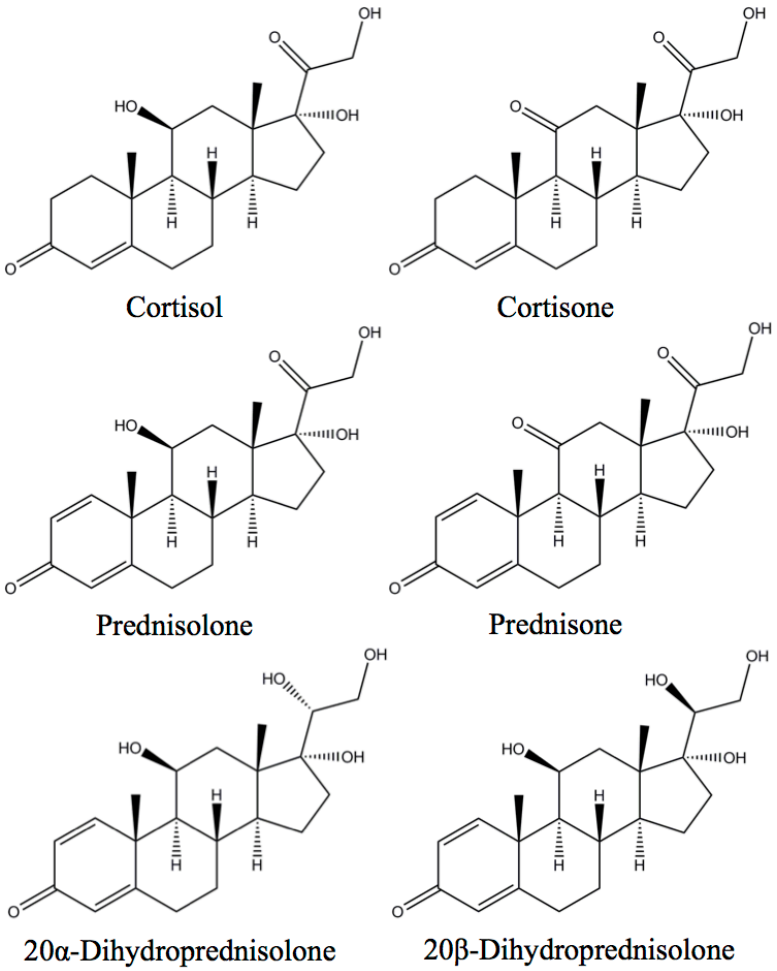

Figure 3.1. Chemical structure of the glucocorticoids under study.

2. Material and methods

2.1. Reagents and chemicals

Standards of prednisolone, prednisone, cortisone, cortisol, dihydrocortisone and methylprednisolone were purchased from Sigma-Aldrich (St. Louis MO, USA). The metabolites 20α-dihydroprednisolone and 20β-dihydroprednisolone were purchased from Steraloids (Rhode Island, USA). Internal standards were cortisol-d_4 (Sigma-Aldrich, USA) and prednisolone-d_8 (TRC, Canada). Reagents were of analytical grade when used for extraction purposes and obtained from VWR International (Merck, Darmstadt, Germany). The reagents were of LC-MS Optima grade for UHPLC-HRMS application. These were obtained from Fisher Scientific UK (Loughborough, UK). Ultrapure water was produced with an Arium 611 UV system (Sartorium Stedim Biotech, Aubagne, France). Isolute C18 (EC) (500 mg, 10 mL) cartridges were purchased

from Biotage (Uppsala, Sweden). Strata-X (200 mg, 6 mL) cartridges were purchased from Phenomenex, Inc. (Torrance, USA). Primary stock solutions were prepared in ethanol at a concentration of 200 µg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at a range of 0.1 - 10 µg mL^{-1}.

2.2. Instrumentation

Analyses were carried out on an UHPLC system, which consisted of an Accela UHPLC pump, an Accela Autosampler and Degasser (Thermo Fisher Scientific, San José, CA, USA). Separation of the glucocorticoids was carried out on a reverse phase Nucleodur C18 Isis UHPLC column (1.8 µm, 100 × 2 mm, Macherey-Nagel, Düren, Germany) at a column oven temperature of 30 °C. The elution gradient was carried out with a binary solvent system consisting of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) at a constant flow rate of 0.3 mL min-1. Optimized separation of all analytes was obtained using a linear gradient starting with a solvent mixture (v/v) of 80% A and 20% B. The percentage of acetonitrile was increased to 25% in 1 min, and held there for 5.0 min. Next, a linear increase to 95% B in 1 min was performed, and further up to 100% in 1 min and held there for 2.0 min. In between samples, the column was allowed to re-equilibrate at initial conditions for 2 min. A 10 µL aliquot of each sample was injected for analysis. High-resolution mass spectrometric analysis was performed on an Exactive™ benchtop mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization probe (HESI II), operating in the polarity switching mode. Ionization source working parameters were optimized and reported in Table 3.1.

Table 3.1. Instrumental parameters used for HESI (II)-ionization of glucocorticoids.

Instrumental Parameter	Value
Spray voltage	4 Kv
Sheath gas flow rate	75 au*
Auxiliary gas flow rate	7 au
Sweep gas flow rate	2 au
Capillary temperature	280 °C
Heater temperature	300 °C
Capillary voltage	45 (-32) V
Tube lens voltgae	95 (-100) V
Skimmer voltage	16 (-20) V

* au: arbitrary units

The resolution was set at 50,000 FWHM at 1 Hz and a scan range of *m/z* 150-650 was chosen. The automatic gain control (AGC) target was set at balanced (1 x e^6 ions) and the High Energy Collision Dissociation (HCD) cell was turned off. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific).

2.3. Samples

Faeces samples were obtained on two different occasions from three adult Holstein-Friesian cows, housed at the Faculty of Veterinary Medicine (Ghent University) in Merelbeke, Belgium. The first three faeces samples were pooled and used during the validation of the analytical method, the second sampling pool was employed as matrix for the stability study. An additional 18 blank faecal samples were used for evaluating the ruggedness and specificity of the analytical method originated from 18 different cows housed in the animal facilities of Centre d'Economie Rurale (CER) (Marloie, Belgium). Faeces was collected by rectal grab sampling, mixed thoroughly by manual stirring and divided into aliquots.

2.4. Sample preparation

2.4.1. Fractional factorial design

Because of the complex nature of faeces, an appropriate extraction and purification procedure is required. A sequential strategy of experimental design was used to optimize the analytical extraction of glucocorticoids from faeces. To screen the dependent variables that might significantly affect the extraction, a Plackett-Burman design was used [32][38]. This design is useful to screen for the main variables from a large number of variables that may affect the response and consists of a two-level design to investigate N-1 variables, with N runs, where N should be a multiple of 4. This Plackett-Burman design is known as a Saturated Main Effect design because all degrees of freedom are utilized to estimate the main effects [39]. The starting point for the development of the extraction procedure consisted of liquid-liquid extraction followed by defatting and clean-up by solid-phase extraction [40][41][42]. Table 3.2. shows the eight variables that were considered as well as the level of each variable used. The excessive variable was set as dummy [43]. For a Plackett-Burman design consisting of 11 variables, 12 experimental runs were sufficient to calculate the main effect of each variable. The experimental design matrix and data analysis were carried out with the software program Modde 5.0

(Umetrics, Umea, Sweden).

Table 3.2. Parameter variable values selected for the Plackett-Burman design.

Variable	Unit	Lower value	Upper value
Qualitative			
Extraction solvent	-	TBME*	Diethylether
2th Extraction	-	Yes	no
Defatting step	-	Yes	no
Solvent for defatting	-	n-Hexane	Petroleum benzine
Type of SPE columns	-	C18 (EC)	Strata X
Quantitative			
Volume of extraction solvent	mL	5	10
Volume 2th extraction solvent	mL	5	10
Time of rotating	min	10	30

*TBME: tert-butyl methylether

2.4.2. Sample extraction

Five grams of pooled faecal material was spiked with the internal standards (cortisol-d_4 and prednisolone-d_8) to obtain a final concentration of 10 µg kg^{-1}. After adding 4 mL of ultra pure water and 2 mL of 10% sodium carbonate buffer, the samples were vortexed for 1 min. A liquid-liquid extraction was performed by adding 10 mL of tert-butyl methylether (TBME). After 10 min of rotating at 1200 x g, the sample was centrifuged at 7600 x g for 10 min at 7 °C. The organic layer was collected and transferred to a 15 mL tube. This liquid-liquid extraction was repeated by adding another 5 mL of TBME to the faecal material. The organic phase was dried under a gentle stream of nitrogen at a temperature of 60 °C and reconstituted in 1.5 mL of methanol and 375 µL of ultra pure water, vortexed for 1 min and twice defatted with 2.5 mL of petroleum benzine prior to the SPE. Prior to sample loading, the C18 (EC) cartridge was conditioned with 5 mL of methanol and 5 mL of water and afterwards washed with 5 mL of water, 5 mL of methanol in water (20:80, v/v) and 2.5 mL of n-hexane. The analytes were eluted with 6 mL of ethyl acetate. Next, the pooled organic phase was dried under a gentle stream of nitrogen at a temperature of 60 °C and reconstituted in 100 µL of eluens, corresponding to the initial mobile phase conditions and transferred to UHPLC-MS vials for analysis.

2.5. Analytical method validation

Validation of the method was performed by adopting the protocol proposed by Antignac *et al.* (2003) [44]. This protocol was tailored for validating analytical methods based on MS detection

and offers a compromise between CD 2002/657/EC [37] and the practical aspects and limitations related to laboratory work. The validation protocol was designed as follows.

Analysis of 18 blank samples, originating from 18 different cows was performed to check the ruggedness and specificity of the method. Pooled faeces of three adult cows was used to evaluate the specificity, selectivity, linearity, precision, limit of detection, limit of quantification, decision limit, detection capacity and mean recovery. Analysis of 20 blank samples of this bovine faecal pool (mixture of three cows) was performed to calculate the endogenous levels of the natural glucocorticoids cortisol, cortisone and the metabolite dihydrocortisone. This permitted to determine the specificity of each compound by calculating the average and standard deviation of the noise amplitude within the retention time window of that compound, expressed relative to the selected internal standard signal amplitude. The calibration curves consisted of eight fortification levels. The linearity and lack-of-fit were evaluated by calculating the regression coefficient (R^2) and by building an univariate linear regression model. The pooled bovine faecal samples were fortified with concentrations, ranging from 1.25 to 50 µg kg^{-1}. To evaluate the precision of the developed analytical method, repeatability and within-laboratory reproducibility were determined by calculating the relative standard deviations (%RSD). For the endogenous compounds, the limit of detection and limit of quantification were determined in 20 unfortified pooled faecal samples, as the lowest level at which a compound could be identified with a signal-to-noise ratio greater than 3 and 10 respectively [45].

2.6. Quality assurance

A mixture of the internal standards cortisol-d_4 and prednisolone-d_8, at a concentration of 10 µg kg^{-1}, was added to every sample, prior to extraction (Table 3.3.). Prior to sample analysis, 10 µL standard mixture of the targeted glucocorticoids at 10 µg L^{-1} was injected to check the operational conditions of the UHPLC-HRMS device. The compounds were identified based on their retention time relative to the retention time of the internal standard of choice and on the $^{13}C/^{12}C$ isotopic ratio according to the criteria described for high-resolution MS in CD 2002/657/EC [37]. After identification, the concentrations of the detected glucocorticoids were calculated by fitting their area ratios into eight-point calibrations curves, set up in bovine faeces. Area ratios were determined by integration of the area of an analyte under the specific

extracted chromatograms in reference to the integrated area of the internal standard.

Table 3.3. UHPLC-HRMS parameters used for the various glucocorticoids with indication of elemental composition, accurate mass, internal standard used, retention time and ionization modus.

Analyte	Elemental composition	Accurate mass (*m/z*)	Internal standard	t_R (min)	Ion mode
Dihydrocortisone	$C_{21}H_{30}O_5$	363.21588	Cortisol-d_4	3.92	+
Cortisol	$C_{21}H_{30}O_5$	363.21588	Cortisol-d_4	5.32	+
Cortisone	$C_{21}H_{28}O_5$	361.20062	Cortisol-d_4	5.66	+
Prednisolone	$C_{21}H_{28}O_5$	361.20062	Prednisolone-d_8	5.09	+
Prednisone	$C_{21}H_{26}O_5$	359.18478	Prednisolone-d_8	5.16	+
Methylprednisolone	$C_{22}H_{30}O_5$	375.21627	Prednisolone-d_8	7.81	+
20α-dihydroprednisolone	$C_{21}H_{30}O_5$	363.21588	Prednisolone-d_8	3.31	+
20β-dihydroprednisolone	$C_{21}H_{30}O_5$	363.21588	Prednisolone-d_8	3.62	+
Cortisol-d_4	$C_{21}H_{27}D_4O_5$	367.24171	-	5.27	+
Prednisolone-d_8	$C_{21}H_{21}D_8O_5$	369.25034	-	4.96	+

2.7. Stability study

During the long-term stability study, the changes in glucocorticoid concentrations were studied under different conditions including multiple freeze-thaw cycles. The glucocorticoids investigated comprised cortisol, cortisone, dihydrocortisone, prednisolone, prednisone and methylprednisolone. Aliquots of five gram of pooled faeces (mixture of three cows) were fortified with a standard mixture of the six glucocorticoids at 20 µg kg^{-1}. In total, three major parameters were investigated: redox conditions, influence of ethanol addition and lyophilization (Figure 3.2.). In the first batch of samples, the stability of glucocorticoids was tested under different redox potentials. To obtain an anaerobe environment the faecal samples were flushed with N_2 during 30 min. The second group consisted of faecal samples to which 10 mL of ethanol was added. In the last part of the study, faecal samples were lyophilized during 4 days to obtain dry faecal matrix. For each time point in the study and for each parameter set, samples were prepared in triplicate and stored respectively at -80 °C, -20 °C, 4 °C and room temperature (15 - 20 °C) up to 20 weeks. At different time intervals, the six glucocorticoids in each batch of faecal samples were quantitatively analysed: 1, 2, 4, 10 and 20 weeks.

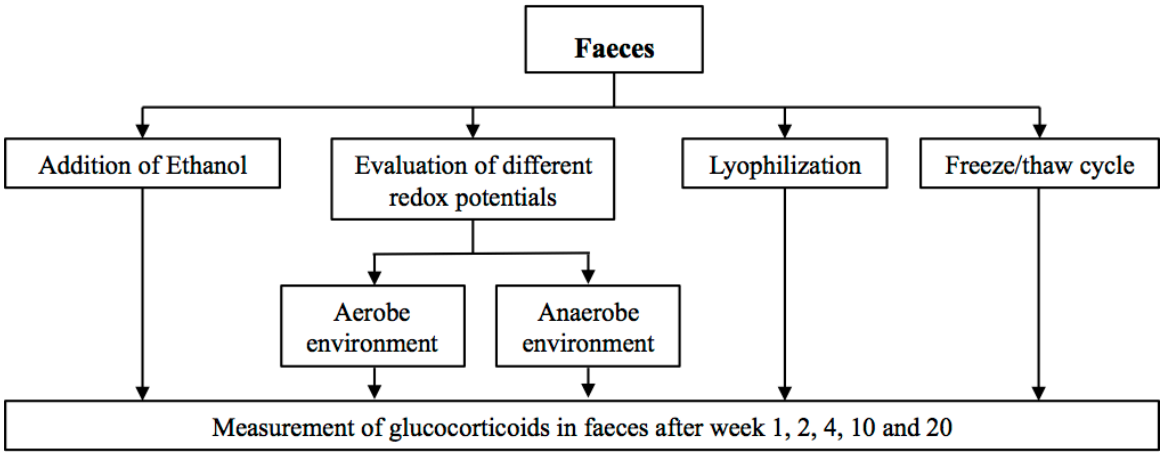

Figure 3.2. Overview of the experimental protocol of the stability study.

Additionally, the effect of freeze/thaw cycles was investigated by consecutively thawing the frozen samples (i.e. remove from freezer and allow to thaw at room temperature) in the first week, refreezing the samples and finally thawing them again for analysis at the next time of sampling (after 2, 4, 10 and 20 weeks).

2.8. Statistical analysis

To evaluate the Plackett-Burman design, a one-way analysis of variance (ANOVA) test was performed using Modde 5.0. Data of the stability study were analysed by multiple linear regression analysis using SPSS Statistics 20 (IBM, United States). A p-value below 0.05 indicated a significant difference.

3. Results and discussion

3.1. Optimization of extraction procedure

Sample preparation is a crucial step towards the unambiguous detection and accurate quantification of glucocorticoids in bovine faeces. For evaluating the effects of eight variables in the extraction procedure, a Plackett-Burman experimental design was employed. Based on literature information, faecal material can contain about 20% fat [40][41] so a defatting step was included. Since in most species, steroids are excreted in faeces in the unconjugated form, no deconjugation step was included in the extraction procedure [11][12][46]. The following variables were esteemed: extraction solvent, volume of the extraction solvent, 2nd extraction, volume of the 2nd extraction solvent, time of rotating, defatting step, solvent for defatting and

type of SPE column (Table 3.2.). The difference between a low and high level was set in this way to obtain a maximal statistical difference. Hence it was possible to identify the most significant variables. The experimental design was executed in a random order.

The critical variables were volume of extraction solvent, whether or not a 2nd extraction was performed and the presence of a defatting step (Supplementary table). These variables had a significant statistical influence ($p < 0.05$) on the extraction efficiency. Further optimization of these categorical variables was not necessary.

For the non-critical variables it was observed that for prednisolone and cortisone, using 10 mL of the extraction solvent TBME displayed a positive regression coefficient, which resulted in an increased recovery. Both SPE procedures gave satisfactory results, but the C18 (EC) cartridge proved more suitable for the intended generic extraction since the 40% loss of metabolites i.e. 20α-dihydroprednisolone and 20β-dihydroprednisolone, was limited during clean-up. For prednisone, cortisol and dihydrocortisone the defatting step showed a significant positive effect on the extraction efficiency. The solvents commonly used for defatting purposes are hexane and petroleum benzine [41]. The best results were obtained after defatting with petroleum benzine.

The most promising settings were included into the extraction procedure (cf. 2.4.2.).

3.2. UHPLC and MS parameters

Chromatographic separation of glucocorticoids poses a true analytical challenge, due to the equal molecular masses of prednisolone and cortisone on the one hand and 20α-dihydroprednisolone, 20β-dihydroprednisolone, dihydrocortisone and cortisol on the other hand, rendering mass spectrometric separation impossible. In a previous study, several UHPLC columns were tested and compared for the chromatographic separation of glucocorticoids in urine, leading to the selection of the Nucleodur C18 Isis (1.8 µm, 100 x 2 mm, Macherey-Nagel) column [34]. The mobile phases were also adopted from this study i.e. 0.1% formic acid in water and 0.1% formic acid in acetonitrile. To achieve chromatographic separation of the isomers 20α-dihydroprednisolone and 20β-dihydroprednisolone the gradient had to be carefully adapted (Figure 3.3.).

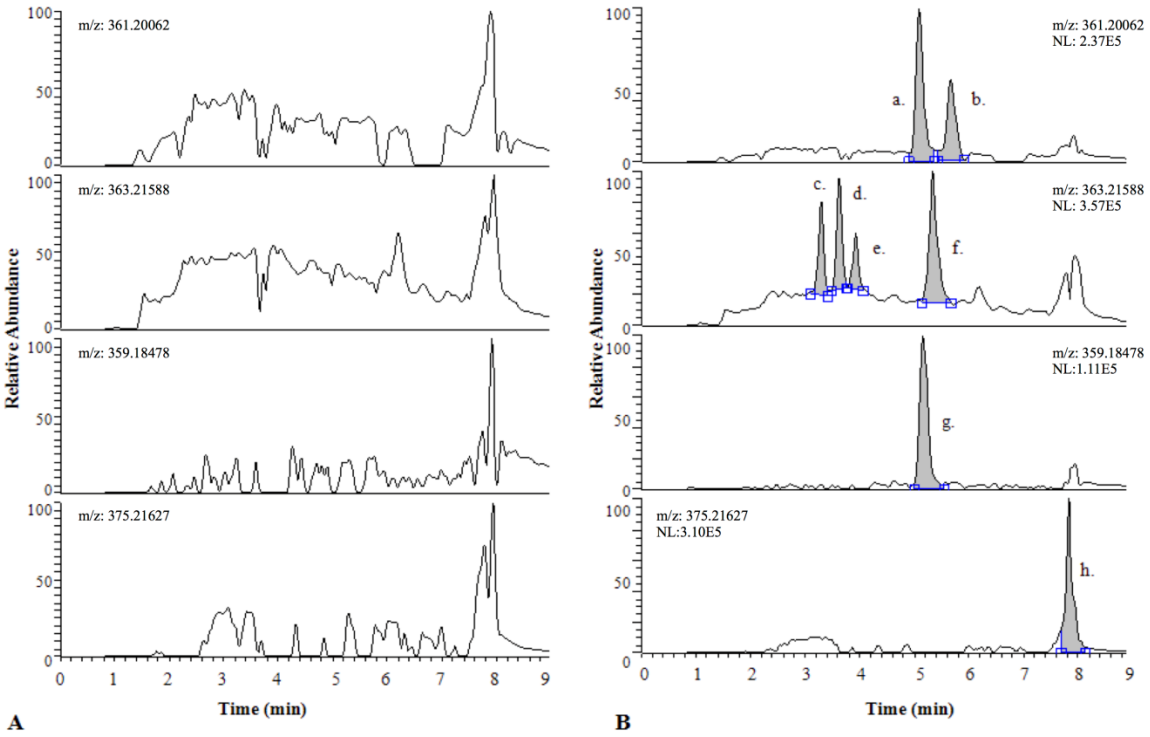

Figure 3.3. Chromatogram of a blank faecal sample, i.e. a sample that was not enriched with glucocorticoid standards (A), and chromatogram of a faecal sample fortified with 5 µg kg^{-1} of (a) Prednisolone (RT: 5.09; MA: 1742418; S/N: 634); (b) Cortisone (RT: 5.66; MA: 1053589; S/N: 333); (c) 20α-dihydroprednisolone (RT: 3.31; MA: 517958; S/N: 43); (d) 20β-dihydroprednisolone (RT: 3.62; MA: 573864; S/N: 32); (e) Dihydrocortisone (RT: 3.92; MA: 304443; S/N: 35); (f) Cortisol (RT: 5.32; MA: 1036409; S/N: 288); (g) Prednisone (RT:5.16; MA: 858750; S/N: 596); (h) Methylprednisolone (RT: 7.81; MA: 1875146; S/N: 698) (B). The maximum mass deviation was set at 4 ppm.

Before determining the optimal MS conditions, for each glucocorticoid standard and deuterium-labeled internal standard (10 µg L^{-1}) the observed masses were compared with the theoretical masses, which were calculated using Xcalibur 2.1, by direct infusion on the HRMS. The mass deviations, expressed in parts per million (ppm) and defined as: 10^6 x [(measured mass – theoretical mass) / theoretical mass], were found to be below 3 ppm. In case of HRMS applications, CD 2002/657/EC states that the presence of the $[M+H]^+$ or $[M-H]^-$ ion with their specified retention times and respective accurate masses is insufficient for the identification and confirmation of the selected analytes since at least two diagnostic ions are requisite, i.e. four identification points for forbidden substances such as corticosteroids [37]. Therefore, the mono-isotopic pattern (^{13}C ion) may assist in the further confirmation of a compound's identity since then two additional identification points may be granted. An isotopic ion was only found suitable

as a diagnostic ion when the corresponding $[M+H]^+$ or $[M-H]^-$ was also detected and the calculated relative ion intensity was in compliance with CD 2002/657/EC requirements [37].

Based on peak intensities, peak areas and signal-to-noise ratios, the optimal HRMS parameters were set for each individual analyte (Table 3.3.). Based on the analysis of fortified faecal samples at different mass resolutions (i.e. 50,000 and 100,000 FWHM) a mass resolution of 50,000 FWHM at 1 Hz was selected. The optimal AGC value was found to be the balanced scan ($1 \times e^6$ ions).

3.3. Method validation

In order to validate the analytical method for quantitative confirmation (CD 2002/657/EC) [37] pooled faeces of three adult cows were used. After homogenization, the performance characteristics of the method were evaluated. Since cortisol and cortisone detection in faeces has been reported [14], the endogenous concentration of 'blank' faeces samples, i.e. samples that were not enriched with glucocorticoids, was measured. In the pooled faeces batch (n = 20) obtained in light of this validation and in 18 faecal samples, originating from 18 different cows, the endogenous concentrations of cortisol, cortisone and the metabolite dihydrocortisone were below LOD and therefore not granted any further consideration during the validation study. These findings are in line with Arioli *et al.* (2010) who state that cortisol and cortisone are frequently below LOD in faeces except for cattle find under stressful situations [47]. During the validation, several variations i.e. variation of time, different persons, different origins of matrices, solvents, stock solutions, etc. were introduced to evaluate the ruggedness of the method.

3.3.1. Specificity

To establish the specificity of the analytical methods, 18 aliquots of pooled faeces samples were fortified with each glucocorticoid standard in order to reach a concentration of 5 µg kg^{-1}. During the additional analysis of 18 faecal samples, originating from 18 different cows no interferences were noticed. The specificity of the analytical approach was confirmed since no interfering peaks with a signal-to-noise of 3 or more were observed at the specific retention times of the targeted glucocorticoids (Figure 3.3.). As a result, the developed method was found to be specific for the validated compounds.

3.3.2. Selectivity

Analytes were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the corresponding internal standard [37]. Besides, when a chromatographic peak of interest had a signal-to-noise of at least 3, the accurate mass of the ion $[M+H]^+$ was taken into account. Within this study, a maximum mass deviation of 4 ppm was allowed.

3.3.3. Linearity

Linearity was evaluated in faeces for each compound by using an unweighted linear regression in the 1.25 - 50 µg kg^{-1} concentration range. Eight-point calibration curves were constructed in pooled faeces. The mean regression coefficients (R^2) of the calibration curves were calculated by plotting area ratio versus concentration. For all target compounds, regression coefficients of 0.9940 or higher were found. This suggests a good linear correlation (Table 3.4.). Additionally the lack-of-fit was assessed by building an univariate linear regression model (SPSS version 21.0) with the triplicate calibration concentrations as independent variable and the respective area ratio as dependent variables. The resulting regression model equations were all reported as linear (F-test; $p < 0.05$; $R^2 \geq 0.99$). Model validity was additionally confirmed by absence of any lack-of-fit (95% confidence interval).

3.3.4. Precision

Evaluation of the precision included the determination of the repeatability and within-laboratory reproducibility of this new method. Both validation parameters were evaluated by calculating the relative standard deviation (%RSD) of three fortification levels (Table 3.4.). These levels were based on the established maximum residue limits (4 µg kg^{-1} and 10 µg kg^{-1} in bovine muscle for prednisolone and methylprednisolone respectively) [5]. Three series of six replicates were analysed to determine repeatability and were carried out on different occasions by the same operator under repeatable conditions. As presented in Table 3.4., the calculated RSD values were below 15%, indicating a good repeatability according to European Criteria 2002/657 [37].

The within-laboratory reproducibility was evaluated with four series of six replicates of samples analysed by two different operators on three different days. The calculated RSD values were below 20%, indicating a comparable precision with the method of Arioli *et al.* (2010) and

Weltring *et al.* (2012) where the RSD values were respectively 5.2 - 16.2 and 5.5 - 9.1 [12][47].

Table 3.4. Decision limits (CC_α) and detection capabilities (CC_β) calculated for the synthetic glucocorticoids in faeces according to 2002/657/EC [37]. Limits of Detection (LOD) and Limits of Quantification (LOQ) calculated for the natural glucocorticoids in faeces. Overview of the main performance characteristics for the eight glucocorticoids, analysed in faeces, according to 2002/657/EC [37].

Analyte	Linearity R^2	LOD[1] CC_α[2] (µg L^{-1})	LOQ[1] CC_β[2] (µg L^{-1})	Nominal concentration (µg L^{-1})	Recovery (%)	Precision	
						Repeatability RSD (%)	Intra-lab. reprod. RSD (%)
Dihydrocortisone	0.99	2.10[1]	5.00[1]	2.5	81	12.39	12.69
				5	100	7.14	18.47
				10	104	5.64	7.67
Cortisol	0.99	0.55[1]	0.70[1]	2.5	99	4.14	6.37
				5	100	2.44	2.75
				10	100	3.90	4.29
Cortisone	0.99	0.55[1]	1.66[1]	2.5	102	3.80	10.00
				5	102	5.11	4.94
				10	101	4.04	4.08
Prednisolone	0.99	0.50[2]	0.65[2]	2.5	103	3.32	6.52
				5	110	7.81	7.28
				10	107	2.75	5.67
Prednisone	0.99	0.15[2]	0.40[2]	2.5	97	7.15	10.93
				5	103	5.48	5.32
				10	106	3.58	5.87
Methylprednisolone	0.99	2.95[2]	3.35[2]	2.5	98	10.55	19.78
				5	103	11.50	13.66
				10	100	6.23	5.92
20α-dihydroprednisolone	0.99	1.35[2]	3.60[2]	2.5	97	8.86	9.48
				5	100	7.40	7.05
				10	109	3.85	6.67
20β-dihydroprednisolone	0.99	1.25[2]	5.20[2]	2.5	92	11.52	13.37
				5	103	11.94	12.12
				10	105	5.82	7.66

1: natural and 2: synthetic glucocorticoids.

3.3.5. Limit of detection and limit of quantification

For the natural glucocorticoids cortisol, cortisone and the metabolite dihydrocortisone, the limits of detection (LOD, S/N ≥ 3) and quantification (LOQ, S/N ≥ 10) were theoretically calculated based on eight-point calibration curves. The LODs ranged from 0.55 to 2.10 µg kg^{-1} and the LOQs ranged from 0.70 to 5.00 µg kg^{-1} (Table 3.4, Figure 3.4.). After theoretically calculating the LOD and LOQ of each compound, these were experimentally confirmed by spiking experiments.

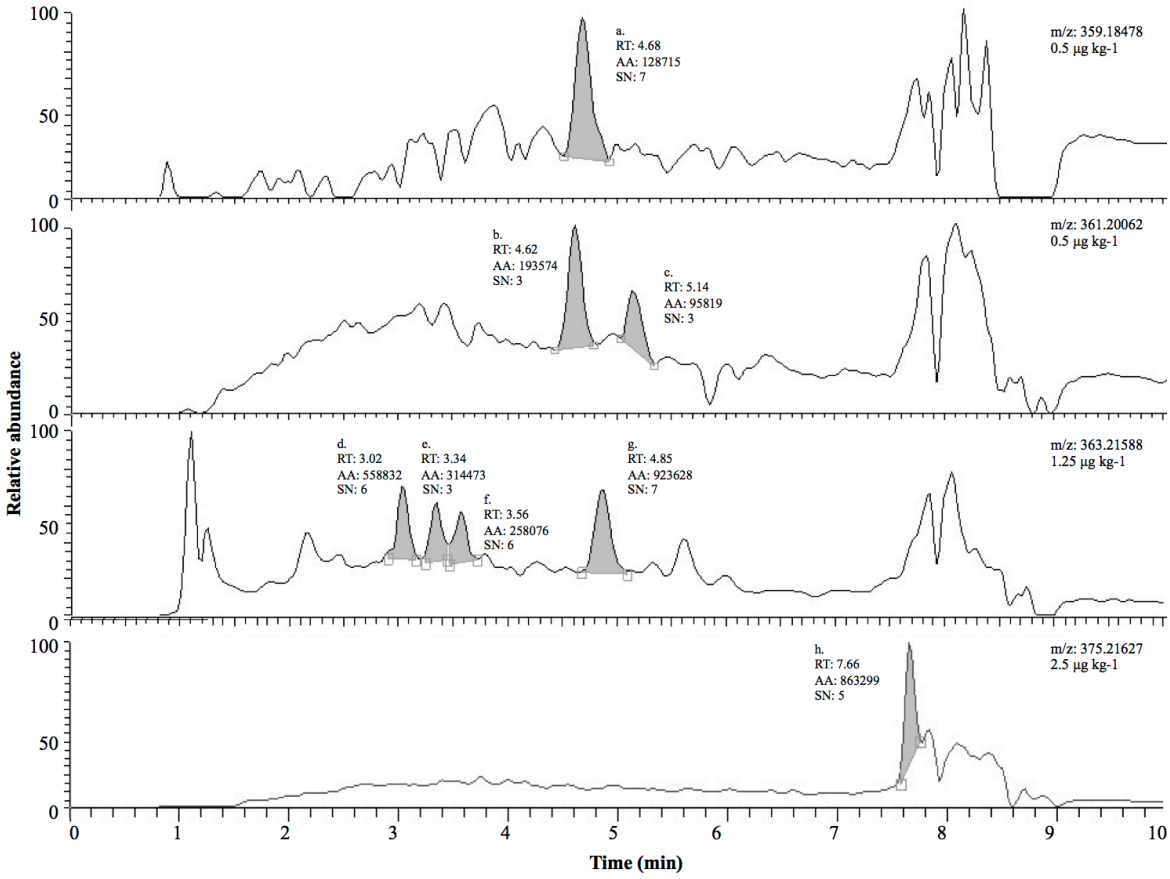

Figure 3.4. Chromatogram of a blank faecal sample fortified with (a) 0.5 µg kg^{-1} prednisone, (b) 0.5 µg kg^{-1} prednisolone, (c) 1.25 µg kg^{-1} cortisone, (d) 1.25 µg kg^{-1} 20α-dihydroprednisolone, (e) 1.25 µg kg^{-1} 20β-dihydroprednisolone, (f) 1.25 µg kg^{-1} dihydrocortisone, (g) 1.25 µg kg^{-1} cortisol, (h) 2.5 µg kg^{-1} methylprednisolone.

3.3.6. Decision limit (CC_α) and detection capacity (CC_β)

For the synthetic glucocorticoids, prednisolone, prednisone and methylprednisolone, blank material is available and therefore the CC_α and CC_β were experimentally determined. The CC_α was calculated as the mean of the noise amplitude plus 2.33 times the standard deviation of the noise amplitude. For calculating CC_β, 20 blank faeces samples were spiked at the determined CC_α level, subsequently the CC_β was determined by calculating the mean concentration at the corresponding decision limit plus 1.64 times the standard deviation of the mean measured concentration [47]. Table 3.4. and Figure 3.4. summarizes the calculated CC_α and CC_β values for the different glucocorticoids. Compared to values reported in literature [47], ranging respectively, from 0.15 to 2.95 µg kg^{-1} and from 0.40 to 5.20 µg kg^{-1}, the calculated decision limits and detection capabilities from this study were in the same order of magnitude.

3.3.7. Mean recovery

As no certified reference material was available, fortified faecal samples were used to determine trueness as the mean corrected recovery. Three fortification levels were considered with six replicates for each level. The calculated mean recoveries ranged from 81 to 110% and were considered to be satisfactory according to CD 2002/657/EC and results found in literature [12][37][47] (Table 3.4.).

3.4. Stability of glucocorticoids in faecal material

The concentration of natural and synthetic steroids may alter by inappropriate storage and the present bacterial activity in faecal matrix. In the present study, the stability of glucocorticoids in bovine faeces was investigated under different experimental conditions.

3.4.1. Effect of temperature during storage

The temperature during preservation can be easily manipulated and controlled for a longer period. Analysis of samples stored for 1, 2, 4, 10 and 20 weeks, demonstrated that the observed glucocorticoid concentrations were highly dependent on the temperature during preservation. For all glucocorticoids, a significant difference ($p < 0.05$) was noticeable between the different temperatures (-80 °C, -20 °C, 4 °C, room temperature). When faecal samples were stored in the freezer (-20 °C and -80 °C), bacterial metabolism is minimized [48]. This effect was noticed for all compounds: after 20 weeks, more than 80% of the initial concentration of cortisol was found at -80 °C. Prednisolone showed an increase of 45% after 20 weeks. Glucocorticoids in faecal samples stored at higher temperatures (4 °C and room temperature) are subjected to higher microbial activity and after 10 weeks in none of the samples glucocorticoids were detected. For the natural glucocorticoid cortisol and the synthetic analogue prednisolone data are presented in Figure 3.5.A. A similar behaviour over time for both compounds was observed.

3.4.2. Effects of lyophilization

During the first 4 weeks, a slight increase in glucocorticoid concentrations was noticed. After 10 and 20 weeks, the concentrations dropped to ca. 80 and 20% of the initial concentration respectively (Figure 3.5.B). These concentration changes were not dependent on preservation temperature, which is in contrast to what previous studies have observed [36][49], where storage at -20°C after lyophilization is recommended.

3.4.3. Effect of adding ethanol

Based on literature findings, faecal samples used for faecal glucocorticoid metabolite analysis are sometimes stored in a preservative solution. Ethanol had been investigated before [49] and showed good results during 14 days. However, these treatments could influence the stability of the glucocorticoids since oxidation and formation of hydroxyl groups on faecal glucocorticoids may occur [33]. Hence, addition of ethanol was included as a test condition in this study. The results showed that the addition of ethanol influenced the extraction efficiency, therefore these results were not discussed in detail. Only the main results are shown in Figure 3.5.C.

3.4.4. Effect of redox potentials

It has been indicated that the microbial population of the large intestinal bacteria of cattle are dominated by strict anaerobes such as *Bacteriodes* spp., *Clostridium* spp., and *Bifidobacterium* spp. [35] and facultative anaerobes i.e. *Eschericia coli*. To investigate their effect on glucocorticoid concentrations upon storage, faecal samples were placed under anaerobe conditions during 20 weeks. These anaerobe conditions exerted significant effects ($p < 0.05$) on the glucocorticoid concentrations when preserved at 4 °C and room temperature. The results are presented for cortisol and prednisolone (Figure 3.5.D). An increase of 40% was observed for prednisolone after 4 weeks at room temperature, which reflects a possible neoformation, induced by facultative anaerobe and anaerobe bacteria present in the faecal matrix.

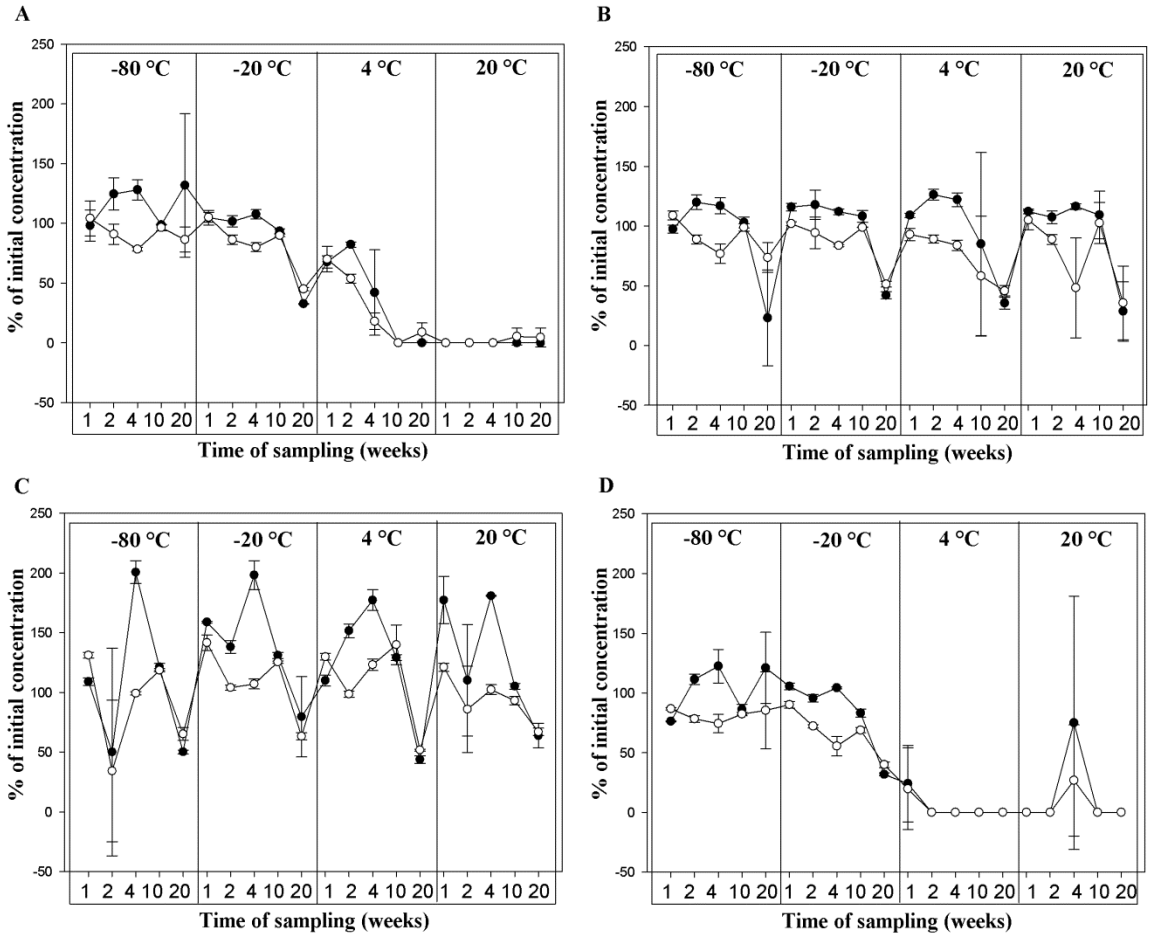

Figure 3.5. Effect of (A) different temperatures on the concentrations of prednisolone (black) and cortisol (white); (B) lyophilization; (C) addition of ethanol; and (D) redox potential during 20 weeks. The mean ± SD of 3 replicates is shown.

3.4.5. Freeze-thaw cycles

Evaluation of freeze and thaw stability was performed by preservation of glucocorticoids in faeces at -20 °C. Each sample was subjected to five freeze-thaw cycles at each round of sampling during the long-term stability study. For the natural glucocorticoids a recovery of 20% was found after the first freeze-thaw cycle. The reason of this decrease is unknown. After the 3th freeze-thaw cycle, a more extensive standard deviation was observed, which clearly demonstrated that the glucocorticoids are affected by the multiple freeze-thaw steps (Figure 3.6.).

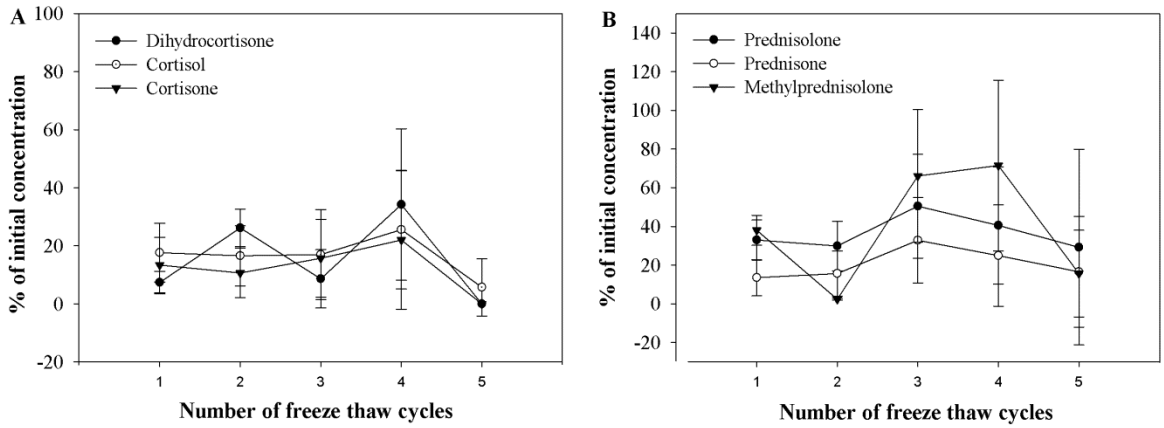

Figure 3.6. Recovery of the different glucocorticoids in faeces exposed to a number of freeze-thaw cycles: (A) natural glucocorticoids and (B) synthetic glucocorticoids. The mean ± SD of 3 replicates is shown.

4. Conclusion

This study describes the successful development and validation of a generic extraction procedure and detection method for glucocorticoids in faeces according to the criteria set in Commission Decision 2002/657/EC [37]. By making use of a sequential strategy of experimental design i.e. Plackett-Burman design, a generic extraction protocol was developed in a more efficient way. This design allows identifying those variables that significantly affect the recoveries of the glucocorticoids from faeces with a reduced number of experiments. The developed HR-Orbitrap-MS method offers a great potential in targeted and untargeted analysis. Because a virtually unlimited number of compounds can be comprised in the detection method while providing accurate mass determination at the same time, it can pursue the perspective of untargeted strategies and retrospective analysis for future experiments.

In conclusion, the long-term stability study (20 weeks) of glucocorticoids in bovine faeces demonstrates freezing (-80 °C) faecal samples without any chemical treatment such as addition of ethanol, increases the recovery of glucocorticoids. The addition of ethanol influenced the extraction efficiency and analysis results of the glucocorticoids and is therefore not recommended. Lyophilization of the faecal matrix proved however a worthy alternative for long-term storage of faecal samples prior to glucocorticoid analysis. Withdrawing water from the matrix, made it possible to store the matrix for long periods (up to 10 weeks) at room temperature without significant ($p < 0.05$) loss of glucocorticoids.

Supplementary table 1. The coefficients and p-values of the compounds in relation with the variables after implementation of a Plackett-Burman experimental design.

	Cortisol		**Cortisone**		**Dihydrocortisone**		**Prednisolone**		**Prednisone**		**Methylprednisolone**	
	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value	Coeff. SC	p-value
Extraction solvent												
TBME	0.1413	0.3076	0.4495	0.1954	0.7092	0.2289	0.03645	0.4867	0.1491	0.005689	0.4166	0.2811
Diethylether	-0.1413	0.3076	-0.4495	0.1954	-0.7092	0.2289	-0.03645	0.4867	-0.1491	0.005689	-0.4166	0.2811
2th Extraction												
Yes	0.2113	0.0150*	-0.4674	0.1810	-0.8208	0.1736	0.09593	0.0105*	0.008501	0.8024	-0.6435	0.1211
No	-0.2113	0.0150*	0.4674	0.1810	0.8208	0.1736	-0.09593	0.0105*	-0.008501	0.8024	0.6435	0.1211
Defatting step												
Yes	54897	0.0304*	-2236	0.6862	20189	0.1558	5804	0.5729	5292	0.0158*	-4184	0.4537
No	-54897	0.0304*	2236	0.6862	-20189	0.1558	-5804	0.5729	-5292	0.0158*	4184	0.4537
Solvent of defatting												
n-Hexane	-1948	0.4921	5520	0.236	-9021	0.0624	-8892	0.1998	159	0.492	-8819	0.0928
Petroleum benzene	1948	0.4921	-5520	0.236	9021	0.0624	8892	0.1998	-159	0.492	8819	0.0928
Type of SPE column												
C18 (EC)	-48658	0.3606	859	0.8763	12729	0.3585	9207	0.3759	427	0.9053	387	0.3922
Strata X	48658	0.3606	-859	0.8763	-12729	0.3585	-9207	0.3759	-427	0.9053	-387	0.3922
Volume of extraction solvent	16481	0.7476	-13920	0.0217*	-8772	0.0413*	-18998	0.0462*	-866	0.8055	692	0.5039
Volume of 2th extraction solvent	902	0.4820	-6395	0.5830	7837	0.5936	1682	0.2959	372	0.3841	836	0.3923
Time of rotation	0.2062	0.1505	-0.5141	0.1407	-0.8688	0.14635	0.0510	0.3312	-0.0572	0.1286	-0.6221	0.1241

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CHAPTER IV:

THE IMPACT OF STRESS ON THE PREVALENCE OF PREDNISOLONE IN BOVINE URINE: A METABOLIC FINGERPRINTING APPROACH

Adapted from:

De Clercq, N., Van Meulebroek, L., Vanden Bussche, J., Croubels, S., Delahaut, P. and Vanhaecke, L. (2015) Journal of Steroid Biochemistry and Molecular Biology doi:10.1016/j.jsbmb.2015.08.026

Abstract

Recent studies support the hypothesis that the glucocorticoid prednisolone can be formed from cortisol under influence of stress. To evaluate this hypothesis, urine samples of supposedly non-stressed bovines (at the farm) and bovines subjected to two different forms of stress, i.e. upon slaughter (natural stress) or following administration of a synthetic analogue of the adrenocorticotropic hormone (pharmacologically-induced increase of cortisol) were analysed, and their urinary cortisol and prednisolone levels evaluated. At the farm, none of the examined samples exhibited urinary prednisolone levels higher than the CC_{α} (0.09 µg L^{-1}). Upon slaughter or following synthetically induced stress, significantly positive correlations between cortisol and prednisolone could be demonstrated, 0.52 and 0.69, respectively. Of all prednisolone-positive urine samples (n = 84), only one showed a prednisolone levels (i.e. 6.45 µg L^{-1}) above the threshold level of 5 µg L^{-1} suggested by the European Reference Laboratories. Subsequently, an untargeted analysis was performed (metabolic fingerprinting) to characterize the urinary metabolite patterns related to the three different cattle groups. In this context, multivariate statistics assigned a total of 169 differentiating metabolites as playing a key role in the urinary pattern in response to stress. Three of these ions were defined as steroids using an in-house created database. As a result, the metabolic fingerprinting approach proved to be a powerful tool to classify unknown bovine urine samples that tested positive for prednisolone, while providing information about the stress status of the animal.

1. Introduction

The main glucocorticoid cortisol and its precursor cortisone have been acknowledged to fulfil a wide range of physiological functions, being amongst others related to stress responses, homeostatic effects, and anti-inflammatory actions [1][2]. Based on these functions, more potent synthetic analogues such as dexamethasone, betamethasone, prednisolone, and methylprednisolone have been introduced and are routinely used in veterinary practice for the treatment of diverse inflammatory diseases and metabolic disorders [3]. Besides their therapeutic actions, glucocorticoids have also been associated with growth-promoting effects, (alone or in combination with anabolic steroids) for fattening purposes as well [4]. However, the therapeutic use of synthetic glucocorticoids is been strictly regulated in the European Union [5] in order to protect consumers against potential harmful residues, present in animal derived food products. More specifically, maximum residue limits (MRLs) have been set for betamethasone, dexamethasone, methylprednisolone, and prednisolone in diverse tissues of animal origin [6]. Moreover, the use of synthetic glucocorticoids is completely prohibited for the sole purpose of increasing the body weight of bovines. For this reason, national residue monitoring plans are implemented in the various member states to detect any misuse of glucocorticoids, whereby urine is considered as the preferred matrix.

In this context, the European Commission declared more non-compliant urine samples for prednisolone in the past few years, which has been reported in their annual Commission Staff Working Document on the implementation of the established national glucocorticoid monitoring plans. Moreover, there was no direct evidence of unauthorized use. These findings, in essence originating from the increased analytical sensitivity of the applied screening methods, led to the hypothesis of an endogenous prednisolone origin. In addition, since most of the prednisolone positive urine samples were collected at the slaughterhouse, it has been suggested that stress may induce the involved metabolic processes [7]. Indeed, various studies report on the detection of prednisolone residues in urine samples that were either collected at the slaughterhouse [7][8][9] or after therapeutic stress inducement [10]. In contrast, during a field survey, no prednisolone was detected in the majority of urine samples from untreated cattle [11]. These observations are supported by the underlying mechanisms, associated with the

physiological stress response. In response to stress, the hypothalamic-pituitary-adrenal (HPA) axis is stimulated whereby the hypothalamus produces corticotropin-releasing hormone (CRH), which in turns triggers the secretion of adrenocorticotropic hormone (ACTH). This latter hormone may affect the adrenal gland, as such promoting the synthesis and release of cortisol [1][12] (Figure 4.1.).

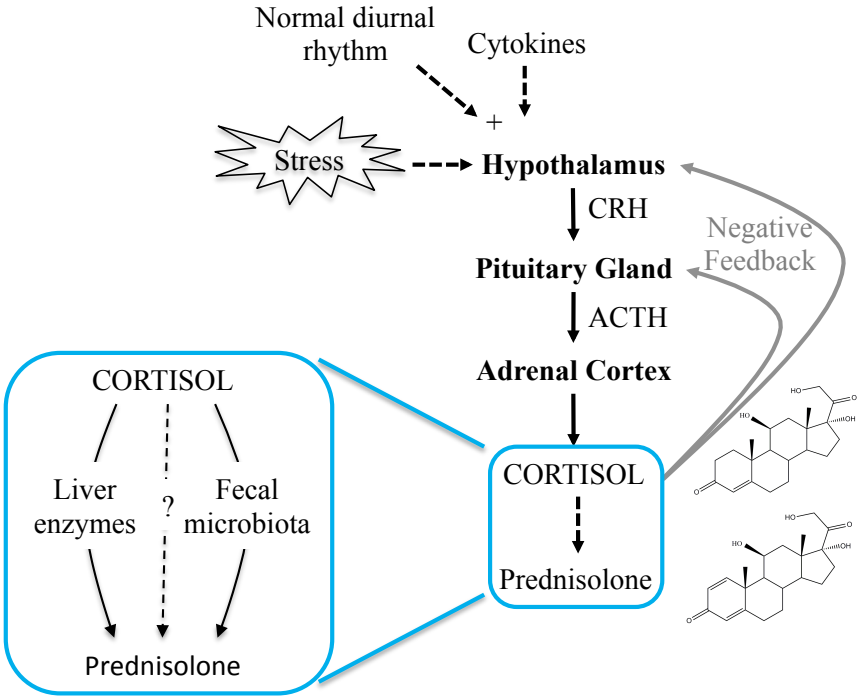

Figure 4.1. Systematic regulation of circulating cortisol levels by the hypothalamic-pituitary-adrenal (HPA) axis and the possible pathways of endogenous prednisolone formation.

Since cortisol (Δ^4-pregnene-11β,17α,21-triol-3,20-dione) is only differing from prednisolone ($\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione) by a single ring double bond, the formation of prednisolone from cortisol may be assumed. This has recently been evidenced by the study of de Rijke *et al.* (2014) [13], whereby a significant decrease of cortisol and formation of prednisolone within 6 h was observed during an *in vitro* incubation experiment with bovine S9 liver enzyme extract. Based on the possible endogenous formation of prednisolone, the European Reference Laboratories have suggested a threshold level for prednisolone in bovine urine of 5 μg L^{-1}, thereby taking into account various potential endogenous origins and influencing factors [13][14].

In this study, the influence of stress on the prevalence of prednisolone and prednisone in bovine urine was evaluated to respond towards the inconsistent data reported in literature and confirm the validity of the above-mentioned threshold level. To this extent, 12 healthy bovines were subjected to a treatment of intramuscular injection with a synthetic analogue of ACTH, i.e. tetracosactide hexaacetate, to induce pharmacologically-induced increase of cortisol. In addition, bovine urine was collected from 144 meat and milk producing bovines under real life conditions differing in degree of stress imposed, i.e. under normal housing conditions at the farm and upon slaughter. The collected urine samples were analysed by usage of full-scan high-resolution Orbitrap mass spectrometry to acquire the samples' metabolic fingerprints, allowing the identification of metabolite patterns that are characteristic for bovines under well-defined stress conditions. Parallel to this untargeted metabolic fingerprinting, a targeted strategy was considered as well, thereby determining the concentration levels of relevant glucocorticoids and derived products; i.e. cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, methylprednisolone, 20α-dihydroprednisolone and 20β-dihydroprednisolone.

2. Material and methods

2.1. Bovine urine collected during ACTH treatment

2.1.1. Test animals

Twelve healthy cows of a mixed breed were subjected to the ACTH *in vivo* study and housed in the animal facilities of the Centre d'Economie Rurale (CER, Marloie Belgium) under controlled experimental conditions. Age of the selected animals ranged from 2 to 6 years and body weight was between 360 and 570 kg. Animals were fed a commercially available diet, commonly applied in zootechnical practice, with *ad libitum* access to water and hay. During the entire study, animals were kept in three separate and equal-sized groups, all housed in a half-covered pen. Prior to the actual ACTH treatment, an initial acclimatization of 18 days was considered. This study was approved by CER's Ethical Committee (CE/Sante/ET/004).

2.1.2. Experimental protocol for ACTH treatment

Subsequent to the acclimatization period, all test animals received at 8 a.m. a daily 2 mg intramuscular (IM) injection of tetracosactide hexaacetate (Pharmacy Department, Faculty of

Veterinary Medicine, Utrecht University), which corresponds to 200 I.U. of ACTH, and this for 4 consecutive days.

2.1.3. Urine sample collection

During the acclimatization period, urine samples were collected daily in the morning at 8 a.m. by a veterinarian with the help of a probe (to prevent faecal contamination) and immediately stored in the dark at -80 °C until analysis [15]. During the ACTH treatment period, urine samples were collected twice a day, one sample prior to IM injection of ACTH and a second 4 h (on the first and second day) or 6 h (on the third and fourth day of ACTH administration) post-administration. Until four days after the termination of the ACTH treatment, daily urine samples were collected in the morning at 8 a.m. (post-treatment samples).

2.2. Bovine urine collected at the farm

2.2.1. Test animals

Urine samples were collected from 11 clinically healthy Belgian Beef cows and 31 milking cows, covering an age range between 5 months and 7 years. The animals were housed at four different farms (CER, n=11; JPW, n=8; PDW, n=12; DD, n=11) located in Belgium, whereby the respective breeders stated that cows had not been subjected to any drug treatment 30 days prior to effective urine sampling.

2.2.2. Urine sample collection

Urine samples were collected during feeding (between 8 and 11 a.m.), thereby waiting for spontaneous micturition, while carefully avoiding fecal contamination. These samples were stored within 2 h at -80 °C in the dark, until analysis.

2.3. Bovine urine collected at slaughter

2.3.1. Test animals

Urine samples at the slaughterhouse (Flanders Meat Group, Zele) were collected from 102 healthy bovines, originating from 43 different farms. The selected animals comprised 64 cows and 38 bulls, all aging between 10 months and 12 years. Although no formal statement with respect to a potential therapeutic pre-treatment could be obtained for all farms, all animals were considered healthy.

2.3.2. Urine sample collection

Urine samples were randomly collected upon slaughter (between 7 and 9 a.m.), according to the order of animal arrival. Urine was thereby obtained from the intact bladder using a sterile needle and stored within 3 h at -80 °C in the dark, until analysis.

2.4. Chemicals and reagents

Analytical standards of prednisolone, prednisone, cortisone, cortisol, dihydrocortisone, and methylprednisolone were purchased from Sigma-Aldrich (St. Louis, USA). The derived metabolites 20α-dihydroprednisolone and 20β-dihydroprednisolone were obtained from Steraloids (Rhode Island, USA). Internal standards were cortisol-d_4 (Sigma-Aldrich) and prednisolone-d_8 (TRC, Toronto, Canada). Primary stock solutions were prepared in ethanol at a concentration of 200 µg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at concentration levels ranging from 0.1 to 10 µg mL^{-1}.

Reagents were of analytical grade when used for extraction purposes and of LC-MS Optima grade for UHPLC-HRMS applications. These reagents were respectively obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK). Ultrapure water was produced with an Arium 611 UV system (Sartorium Stedim Biotech, Aubagne, France).

2.5. Sample preparation

A detailed description of the analytical procedure for glucocorticoid extraction and purification of bovine urine has been described in earlier work [15]. In brief, a five-mL aliquot of urine was enriched with internal standards (cortisol-d_4 and prednisolone-d_8) to reach concentration levels of 10 µg L^{-1}. Next, a twofold liquid-liquid extraction with pure tert-butyl methylether was performed. The organic phases were collected, pooled and dried under a gentle stream of nitrogen at a temperature of 50 °C. The residue was dissolved in 100 µL of solvent, reflecting the initial mobile phase conditions, and transferred to a vial for UHPLC-HRMS analysis.

2.6. UHPLC-Orbitrap-MS

Analysis of the urine extracts was performed by UHPLC-Orbitrap mass spectrometry, according to De Clercq *et al.* (2013) [15]. The chromatographic separation was achieved using an Accela UHPLC system (Thermo Fisher Scientific, San José, USA), equipped with a Nucleodur Isis C18 column (1.8 µm, 100 mm x 2 mm, Macherey-Nagel, Düren, Germany). The binary solvent system

consisted of 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile (80/20, v/v) at a constant flow rate of 300 μL min^{-1}. High-resolution mass spectrometric analysis was performed on an Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionization probe (HESI-II), operating in polarity switching mode. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific). This method has been validated according to Commission Decision 2002/657/EC with a CC_α of 0.09 μg L^{-1} and CC_β 0.37 μg L^{-1} for prednisolone [16].

2.7. Quantitation and normalization

Absolute quantitation of the targeted glucocorticoids and related metabolites (i.e. cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, methylprednisolone, 20α-dihydroprednisolone and 20β-dihydroprednisolone) was established by usage of eight-point matrix-matched calibration curves. In this regard, urine collected before ACTH treatment, containing no residues of prednisolone, prednisone and their metabolites, was used. For cortisol, cortisone and dihydrocortisone, resulting calibration curves were adjusted for the endogenously present concentration levels, which were determined as the average of 5 non-enriched urine samples. Adjusted calibration curve concentration levels ranged from 0.50 to 75 μg L^{-1} for the various targeted metabolites and were constructed by means of peak area ratios (i.e. ratio of analyte to internal standard peak area). Untargeted metabolites were relatively quantified by using the peak abundances.

Normalization of the determined relative and absolute quantitative values was performed based on the creatinine content, which allowed to account for variation that may occur in individual excretion patterns and daily fluid consumption [17].

2.8. Statistical analysis

Equal of variances was verified by Levene's Test and normality of the various data sets by the Kolmogorov-Smirnov method whereas statistical differences were evaluated using one-way ANOVA and post-hoc Tukey's multiple comparisons test (p-value ≤ 0.05). The significance of correlation between two variables was tested using Pearson's correlation coefficients and multiple linear regression analysis (p-value ≤ 0.05) (SPSS™ statistics 21).

2.9. Metabolic fingerprinting of urine samples

First the metabolic fingerprints were established for the acquired full-scan data files by using Sieve™ 2.1 software (Thermo Fisher Scientific, San José, USA), thereby aiming at the characterization of the detected metabolite ions in terms of signal abundance, *m/z*-value and retention time. Metabolite screening was separately performed for the positively and negatively charged ions, on the basis of optimized parameter settings, including an *m/z* scan range of 150 - 650 Da, an *m/z* width of 10 ppm, a retention time range from 1.0 – 9.5 min, a peak intensity threshold of 50,000 arbitrary units, a maximum peak width of 0.5 min, and a maximum number of 30,000 frames. In addition, peak alignment was applied whereby corrections for inherent chromatographic variability during analysis were made. The generated data matrix of ion abundances was normalized by the signal intensities of two deuterium-labeled internal standards i.e. cortisol-d_4 and prednisolone-d_8, which were supplemented prior to extraction and by creatinine content [17].

Subsequently, multivariate data analysis was performed using SIMCA™ 13 software (Umetrics, Malmö, Sweden), thereby aiming to reveal differences in the urinary profiles that are due to different stress conditions. For this purpose, the normalized data matrix was used and pre-processed by logarithmic transformation and center scaling in order to induce normality and standardize the range of metabolite ions, respectively. First orthogonal partial least squares discriminant analysis (OPLS-DA) was applied to model the three different stress conditions, reflected by the urinary metabolic fingerprints of the samples collected from the farm (FARM), at slaughter (SLAUGHTER) and during ACTH treatment (ACTH). Validation of the constructed models was performed by CV-ANOVA (p-value < 0.05), permutation testing (n = 150), and the three model characteristics $R^2(X)$, $R^2(Y)$, and $Q^2(Y)$, calculated by 7-fold cross-validation with a $Q^2(Y)$ of 0.5 indicating good model predictability [18]. Based on the validated models, a selection of relevant metabolites, significantly contributing towards the discrimination of the various stress conditions, was performed. To reveal the significance of the various metabolite ions during the different stress conditions, three different plots were created. First, the Variable Importance in Projection (VIP) plot was created to characterize the discriminating power of each metabolite [19]. In addition, S-plots were constructed, in which the relationship between

covariance and correlation among the OPLS-DA results was visualized. Finally, an extension of the S-plot, the shared and unique structure (SUS)-plot, was generated. By combining the selected ions in each of the used tools, the discriminative ions were determined'

Next, principal component analysis (PCA) and hierarchical cluster analysis (HCA) (single linkage) were performed based on the metabolite profile to reveal similarities among samples and clustering according to the concerned stress conditions.

Subsequently, to investigate the role of steroidal compounds in this process, the retained metabolites were screened against an in-house created database, which comprised the elemental composition of 1693 steroidal compounds (based on the 11th catalogue edition of steroids from Steraloids Inc.). For this purpose, the software programme ToxID 2.1.2 (Thermo Fisher Scientific) was used, thereby setting a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm.

3. Results

3.1. Natural glucocorticoids in bovine urine

3.1.1. Supposedly non-stressed cattle

To evaluate the presence of prednisolone residues in random farm animals under normal housing and assumed stress-free conditions, urine samples of animals from four different farms were analysed. The urinary cortisol levels ranged from 0.349 to 5.81 µg L^{-1} (average 1.80 µg L^{-1}), cortisone levels from 0.437 to 8.37 µg L^{-1} (average 2.49 µg L^{-1}) and dihydrocortisone levels from 0.176 to 10.31 µg L^{-1} (average 2.83 µg L^{-1}). The observed concentration levels were independent of the farm (p-value > 0.05) (Figure 4.2.).

The urinary concentration levels of the animals of the ACTH trial during the acclimatization period (i.e. no stress), ranged for cortisol from 0.411 to 4.26 µg L^{-1} (average 1.79 µg L^{-1}), for cortisone from 0.472 to 5.53 µg L^{-1} (average 2.68 µg L^{-1}) and for dihydrocortisone from 0.222 to 9.36 µg L^{-1} (average 2.27 µg L^{-1}) (Figure 4.2.). No significant differences (p-value > 0.05) in urinary natural glucocorticoid concentrations could be detected between the two groups of supposedly non-stressed cattle (i.e. farm and pre-ACTH).

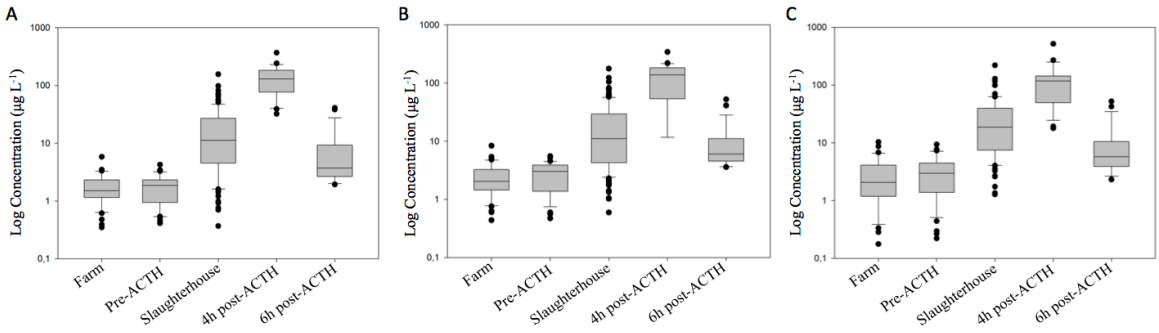

Figure 4.2. Box plot representing cortisol (A), cortisone (B) and dihydrocortisone (C) concentrations in bovine urine collected at the farm, before ACTH administration (pre-ACTH), at the slaughterhouse and during ACTH administration (4h and 6h post-ACTH administration).

3.1.2. Naturally stressed cattle

All urine samples collected at the slaughterhouse contained cortisol (average 18.9 µg L^{-1}, range 0.368 – 155 µg L^{-1}), cortisone (average 21.8 µg L^{-1}, range 0.593 – 177 µg L^{-1}) and dihydrocortisone (average 30.5 µg L^{-1}, range 1.28 – 218 µg L^{-1}). Hereby, it was verified that the cortisol and cortisone levels were independent of animal age or farm of origin (p-value > 0.05). Although the male animals had lower mean cortisol levels than the females, 11.9 µg L^{-1} and 23.1 µg L^{-1}, respectively, this could not be linked to age (p-value > 0.05) (Table 4.1.).

Table 4.1. Urinary concentrations (mean ± SD) of cortisol and prednisolone in cattle at slaughter, with indication of gender and age.

Age (Year)	Bulls				Cows			
	N°	Cortisol	N°	Prednisolone	N°	Cortisol	N°	Prednisolone
0	5	1.83 ± 0.948	0	NF	0	NF	0	NF
1	24	12.9 ± 14.3	4	0.654 ± 1.06	2	78.8 ± 109	2	1.22 ± 1.59
2	5	21.1 ± 11.7	3	0.157 ± 0.0633	3	24.1 ± 28.4	3	0.228 ± 0.0227
3	1	14.1	0	NF	7	19.7 ± 23.9	2	0.623 ± 0.408
4	0	NF	0	NF	16	17.5 ± 17.7	4	0.281 ± 0.104
5	1	3.50	0	NF	14	23.6 ± 30.1	8	0.379 ± 0.284
6	0	NF	0	NF	7	24.4 ± 13.9	4	0.178 ± 0.0614
7	0	NF	0	NF	7	20.7 ± 17.1	3	0.264 ± 0.136
8	0	NF	0	NF	3	12.1 ± 10.2	1	0.1422
9	0	NF	0	NF	4	34.8 ± 26.9	1	0.109
10	0	NF	0	NF	0	NF	0	NF
11	2	NF	0	NF	1	11.7	1	NF
Total	**36**	**11.9 ± 13.1**	**7**	**0.441 ± 0.800**	**64**	**23.1 ± 27.1**	**29**	**0.360 ± 0.440**

3.1.3. ACTH treatment

During ACTH treatment, the urinary cortisol, cortisone and dihydrocortisone levels increased significantly (p-value ≤ 0.05) after 4 h (Day+1 and Day+2) to values ranging from respectively 38.2 to 368 µg L^{-1}, 41.5 to 342 µg $L^{-1}$and 37.3 to 515 µg L^{-1}. After 6 h (Day+3 and Day+4), urinary concentration levels were still significantly higher (p-value ≤ 0.05) compared to the acclimatization period, although less pronounced than after 4 h (Figure 4.2.). The concentrations of cortisol, cortisone and dihydrocortisone 24 h post-treatment were similar to those before tetracosactide hexaacetate treatment.

3.2. Synthetic glucocorticoids in bovine urine

3.2.1. Supposedly non-stressed cattle

Prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone could not be detected in any of the urine samples that were collected at the farm or during the acclimatization period prior to the tetracosactide hexaacetate treatment.

3.2.2. Naturally stressed cattle

In 36 of the 102 examined urine samples collected at slaughter, prednisolone residues were detected at a level higher than the CC_α, whereby the average concentration was 0.391 µg L^{-1} (range 0.900 – 2.35 µg L^{-1}) (Figure 4.3.). Moreover, for some of the remaining urine samples (33 out of 102 analysed samples), prednisolone was detected, but at very low signal intensities, corresponding to calculated concentration levels below the CC_α. These samples were therefore not considered as prednisolone positive. Only 19.4% of the steers were positive for prednisolone compared to 45.3% of the cows, although no significant differences (p-value > 0.05) in urinary prednisolone concentration could be detected.

In 59.8% of the analysed samples (61/102) prednisone could be detected at an average concentration of 1.49 µg L^{-1}, ranging from 0.137 to 10.0 µg L^{-1}. The metabolites 20α-dihydroprednisolone and 20β-dihydroprednisolone could not be detected in any urine sample (Figure 4.3.).

The 95% confidence interval for the proportion of prednisolone positive urine samples (i.e. the number of prednisolone positive urine samples at the slaughterhouse) ranged between 26.9% and 45.6%.

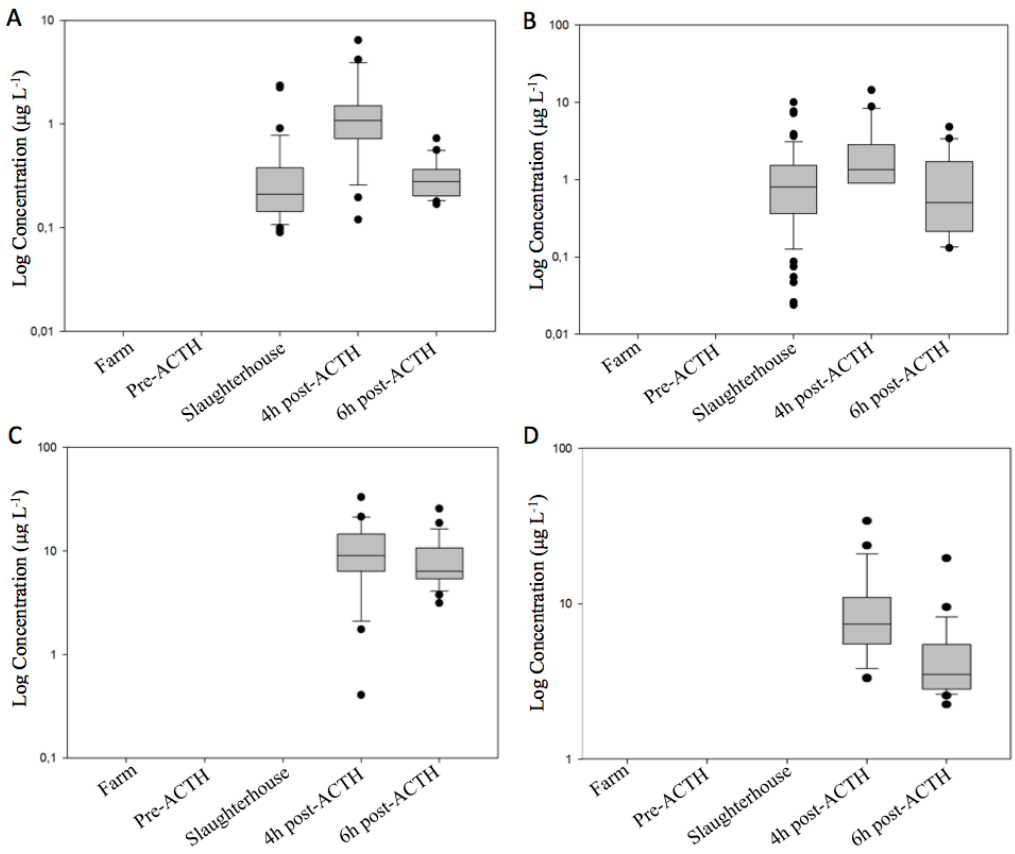

Figure 4.3. Box plot representing prednisolone (A), prednisone (B), 20α-dihydroprednisolone (C) and 20β-dihydroprednisolone concentrations in bovine urine collected at the farm, before ACTH administration (pre-ACTH), at the slaughterhouse and during ACTH administration (4h and 6h post-ACTH administration).

3.2.3. ACTH treatment

Prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone were consistently detected in urine 4 h after IM injections (Day+1 and Day+2). Prednisolone levels in urine collected 6 h after injection were significantly lower (p-value ≤ 0.05) (Day+3 and Day+4) compared to 4 h, although still detectable (Figure 4.3.).

3.3. Correlation between natural and synthetic glucocorticoids during stress

The correlation between cortisol, prednisolone and both their metabolites was calculated using Pearson's correlation. In general a Pearson's correlation higher than 0.7 is considered as strong, while 0.3 to 0.7 is considered as weak and 0 to 0.3 implies no or little correlation [8]. Our results indicated a strong correlation between cortisol, cortisone and dihydrocortisone and between prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone upon ACTH treatment and after slaughter (Table 4.2.).

Table 4.2. Pearson's correlation between the urinary levels of cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, 20α-dihydroprednisolone (20α-DHP) and 20β-dihydroprednisolone (20β-DHP) of urine samples collected upon ACTH treatment and slaughter.

	Correlations					
	Cortisone	Dihydrocortisone	Prednisolone	Prednisone	20α-DHP	20β-DHP
A. ACTH treatment						
Cortisol	**0.960****	**0.931****	0.695**	0.566**	0.508**	0.693**
Dihydrocortisone	**0.915****	1	**0.751****	0.656**	0.599**	**0.746****
Prednisolone	0.671**		1	**0.820****	**0.850****	**0.853****
Prednisone	0.568**			1	**0.919****	**0.828****
20α-DHP	0.485**				1	**0.855****
20β-DHP	0.657**					1
B. Urine samples collected at slaughter						
Cortisol	**0.983****	0.440**	0.521**	0.676**	0.124	0.151
Dihydrocortisone	0.386*	1	-0.212	0.450**	0.142	0.180
Prednisolone	0.537**		1	**0.717****	**0.726****	**0.763****
Prednisone	0.642**			1	**0.801****	**0.829****
20α-DHP	0.201				1	
20β-DHP	0.183					1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

3.4. Metabolic fingerprinting of urine samples

In this study, it was aimed to correctly classify animals according to the imposed stress condition, thereby solely using the urinary metabolite fingerprints. For this purpose, a metabolic fingerprinting approach was carried out, whereby ions were detected and characterized by Sieve^{TM}. The established fingerprints comprised 16269 positively and 11366 negatively charged monoisotopic ions (^{13}C isotope ion species were excluded). The associated data matrices, mainly including the ions' abundances, were then normalized by usage of the signal intensities of two deuterium-labeled internal standards i.e. cortisol-d_4 and prednisolone-d_8, which were supplemented prior to extraction and by creatinine abundances, which reflect urinary densities.

With regard to the subsequent multivariate data analysis, it was in first instance envisaged to reveal any clustering among urine samples that relates to the stress condition. More specifically, supervised OPLS-DA modeling was applied to highlight metabolite ions with a leading role in class discrimination. For this purpose, quantitative data (peak abundances) of 129 randomly selected urine samples, which were collected at the farm (n = 40, FARM), the slaughterhouse (n = 67, SLAUGHTER), or during ACTH treatment (n = 22, ACTH). With respect to the latter, it should

be noted that only urine samples, collected at 4 h post-ACTH administration, were used in the ACTH-group. These data were center-scaled and logarithmically transformed. Two-class OPLS-DA models were constructed with FARM as the common reference class, i.e. the models aimed at discriminating between FARM and SLAUGHTER (i), and between FARM and ACTH (ii) (Figure 4.4.A).

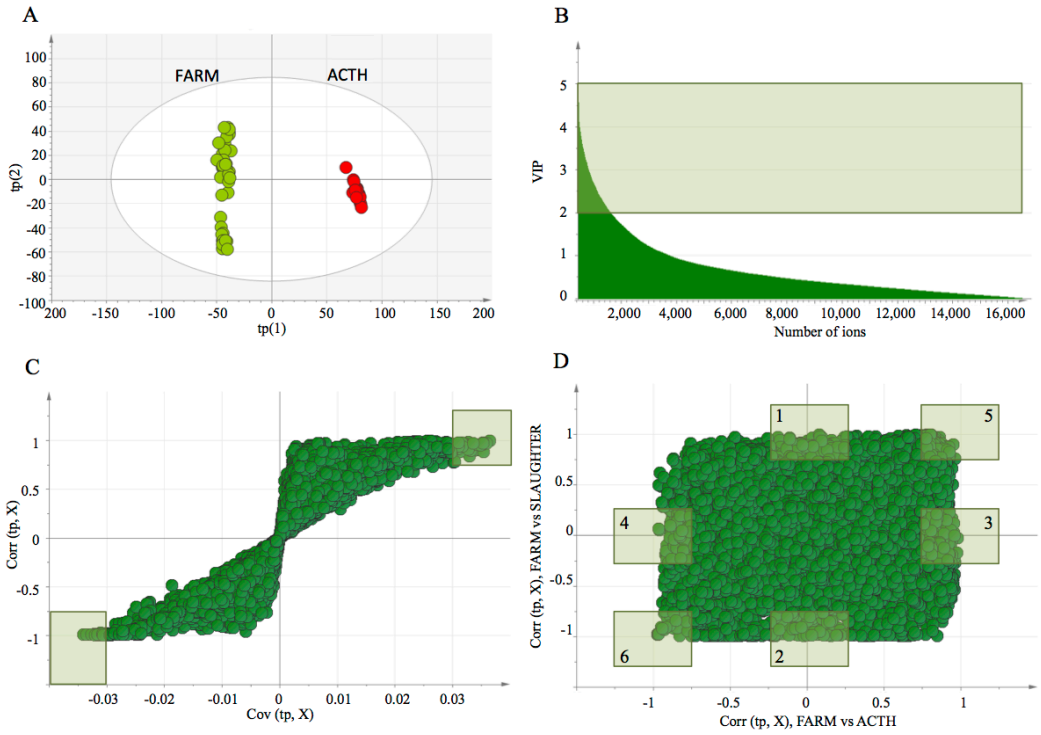

Figure 4.4. OPLS-DA model output to discriminate between the metabolic fingerprints of FARM and ACTH (A). Selection of relevant discriminating metabolite ions was performed for each of the validated two-class models by using VIP-scores (B), S-plot (C) and SUS-plot (D). Shaded areas represent the ions that were considered relevant and retained.

Quality of the constructed models was evaluated by CV-ANOVA (p-value ≤ 0.05), permutation testing, and the model characteristics $R^2(X)$, $R^2(Y)$, and $Q^2(Y)$. With respect to the model characteristics, the following values were obtained (considering either the positive or negative ions, respectively): (i) 0.513, 0.997 and 0.984, (ii) 0.463, 0.985 and 0.975; and (i) 0.571, 0.978 and 0.965, (ii) 0.425, 0.988 and 0.983. Based on the various validation parameters, overall good model predictability was concluded [20]. Selection of discriminating metabolites was in first instance based on the VIP score (Figure 4.4.B), which describes the relative importance of each metabolite ion towards the OPLS-DA based class separation. Generally, a VIP-score > 1 is associated with a significantly discriminating ion [19]. However, given the large number of ions,

enclosed by the metabolite fingerprints, a more rigorous threshold of 2 was adopted in this study. The second selection step focused on both the contribution and reliability of each metabolite ion towards the predictive model component and is visualized by the S-plot [21][22]. In this context, metabolite ions with cut-off values of $|p_1| \geq 0.03$ and $|p_{(corr)}| \geq 0.75$ were considered as relevant and reliably differentiating metabolites [22] (Figure 4.4.C). An extension of the S-plot is the SUS-plot (Figure 4.4.D). This plot was applied to combine the correlation p(corr) of the predictive component from each model, i.e. (i) and (ii), compared to a common control, i.e. FARM. In this plot, the position of an ion relative to the diagonal is indicative for its importance towards each of the various classes, which is interesting to find shared as well as unique metabolite ions [23]. The ions with a unique contribution towards model (i) or model (ii) are located close to either the X- or Y-axis, respectively, when $|p_{(corr)}| \geq 0.75$. In contrast, ions with shared effects are situated close to the plot's diagonal. As such, six areas in terms of metabolite abundance can be defined within the SUS-plot (exemplified in Figure 4.4.D): (1) increased for ACTH only, (2) decreased for ACTH only, (3) increased for SLAUGHTER only, (4) decreased for SLAUGHTER only, (5) increased for both SLAUGHTER and ACTH, (6) decreased for both SLAUGHTER and ACTH. This revealed a total of 169 metabolites (Supplementary table 4.1.), which were thus assigned discriminative value towards the imposed stress conditions as their urinary concentration levels were significantly altered in response to stress compared to the assumed stress-free conditions at the farm.

PCA-modeling indicated the potential of the presented profiling strategy. Hereby quantitative data regarding the 169 differentiating metabolites were gathered for urine samples collected before the first ACTH treatment (ACTH pre-treatment) and the three different stress conditions (FARM, ACTH and SLAUGHTER). Good performance of the PCA-models, separately generated for the positively and negatively charged ions, was indicated by the validation parameters $R^2(X)$, representing the explained variation in X and $Q^2(X)$, which represents the goodness of prediction calculated by 7-fold cross validation. The obtained values were, for positive and negative ionization respectively, 0.752 and 0.635, and 0.754 and 0.669. This PCA-modeling revealed four main sample clusters (Figure 4.5.A), which were confirmed by unsupervised HCA (Figure 4.5.B).

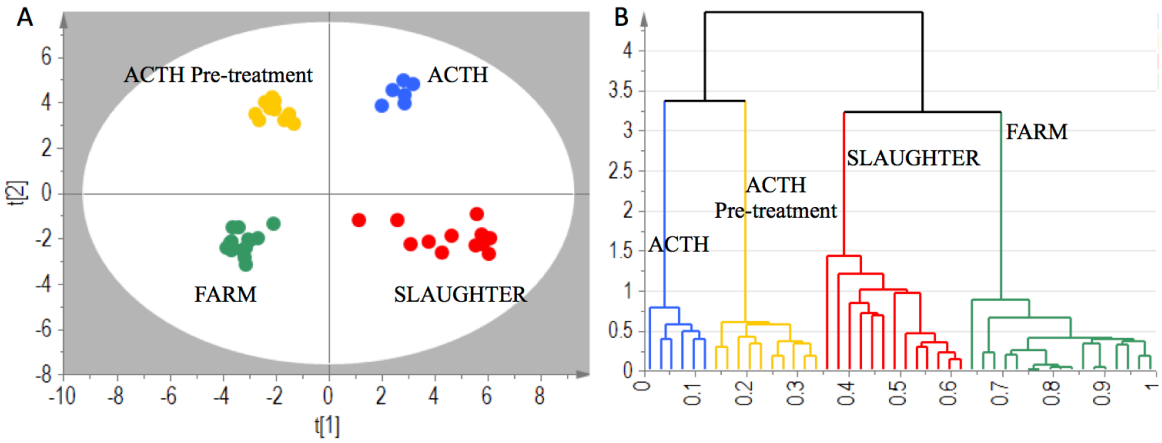

Figure 4.5. Principal component analysis score plot for urine samples and their inherent metabolite fingerprints, representing different stress conditions. (A). Dendrogram enclosing information about the similarities of samples (i.e. clusters) (B).

This HCA clustering projects sample similarities on a two-dimensional diagram (i.e. dendrogram) and allowed to conclude that the observed clustering strongly related to the imposed stress condition. In addition, the accuracy of linking unknown samples to their true stress condition was evaluated by means of a misclassification matrix. With this approach, a matrix is generated by which a comparison can be made between the true and predicted class [24], i.e. stress condition. An independent test set, consisting of 5 urinary fingerprints for each stress condition, was correctly classified, whereby the true class equals the predicted class and a 100% prediction score was achieved.

To visualize the effects of the various stress conditions on the revealed discriminating metabolites, heat map plots were constructed (Figure 4.6.), whereby coloration was performed according to the metabolite ions' intensity levels.

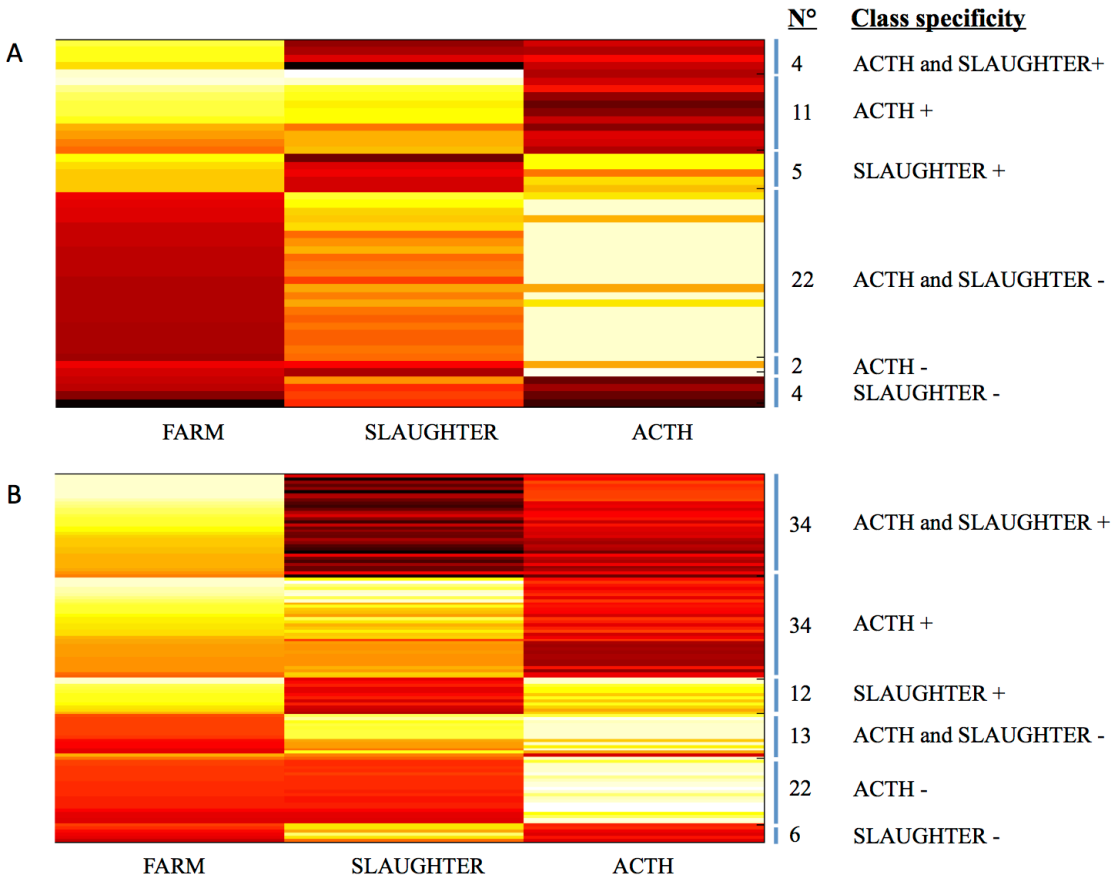

Figure 4.6. Heat map representing the 169 differentiating ions (rows) grouped by sample type (columns). The ion intensities of each ion were averaged along samples and then log transformed. Shades of red and yellow represent elevation and decrease of a metabolite, respectively, relative to the median metabolite level. Right are the number of differentiating ions indicated that are significantly different ($p < 0.05$) with at least one group of sample types and the class specificity of the different ions for the positively (A) and negatively charged ions (B).

These heat maps showed that 38 ions were upregulated and 35 ions were downregulated when animals were under stress, independently of the origin of stress. Besides these 'shared' metabolite ions, 96 metabolites were assigned significant importance towards separation of the various stress origins. More specifically, 45 ions were found upregulated during ACTH treatment compared to the samples collected at the farm or upon slaughter. After evaluating the Pearson's correlation, 11 of these 45 ions were positively correlated (p-value ≤ 0.05) with urinary cortisol and/or prednisolone concentration levels (Supplementary table 4.1., in bold). For the urine samples collected at slaughter, in total 17 ions were upregulated and 10 ions were downregulated compared to the control samples collected at the farm or during ACTH treatment. Moreover, no correlations (p-value > 0.05) with urinary cortisol or prednisolone

levels were noticed for these particular metabolites.

Subsequently, the 169 discriminating metabolites were screened against an in-house created steroidal database, by using the software programme ToxID 2.1.2. Three compounds could be tentatively identified based on the presence of two diagnostic ions: the $(M+H)^+$ ion and the corresponding ^{13}C isotopic ion (Table 4.3.). However, unequivocal identification could not be guaranteed because of the presence of isomers, which may lead to the detection of multiple chromatographic peaks. Taking these findings into account, ToxID enabled a first screening for relevant steroidal compounds.

Table 4.3. Steroidal compounds detected in the urine samples. The software program ToxID was applied for screening of steroidal compounds for which a minimum peak intensity of 1000 and a maximum mass deviation of 5 ppm were used.

Elemental composition	RT (min)	Measured accurate mass	Mass error (ppm)	Polarity	Observed isotope ratio (%)	Influence	Tentative name
$C_{27}H_{44}$	8.54	369.35131	-0.536	+	29.2	SLAUGHTER+	2,4-cholestadiene 3,5-cholestadiene 4,6-cholestadiene
$C_{33}H_{38}O_4$	8.40	499.27923	0.93	+	35.7	ACTH-	5-androsten-3β, 16β-diol dibenzoate
$C_{33}H_{40}O_4$	8.79	501.29437	1.42	+	35.7	ACTH+	5-androsten-3β, 17β-diol dibenzoate 5α-androstan-3α, 17β-diol dibenzoate 5α-androstan-3β, 17α -diol dibenzoate

4. Discussion

In the present study, bovine urinary levels of cortisol, prednisolone and their main metabolites were evaluated in cattle under three different conditions: supposedly non-stressed (at the farm), natural stress (at the slaughterhouse) and pharmacologically-induced increase of cortisol (upon administration of tetracosactide hexaacetate). The baseline cortisol, cortisone and dihydrocortisone levels in non-stressed animals and prior to first tetracosactide hexaacetate treatment were similar. In both situations neither prednisolone nor its metabolites could be detected in any urine sample, which is in line with the results of the survey study performed by Vincenti *et al.* (2012) [11].

The way livestock is handled, transported, restrained and slaughtered can induce stress, which results in elevated cortisol, cortisone and dihydrocortisone concentrations [25]. In our study, only in 5 urine samples, cortisol levels above 70 μg L^{-1} occurred. This could be an indicator of rough handling [26]. On the other hand, cortisol levels close to the baseline indicated that the procedure was either non-stressful or very fast, since it takes 10 to 20 min to reach elevated cortisol levels [27]. Besides, it is important for meat quality that animals are not stressed prior to slaughter to avoid unnecessary depletion of muscle glycogen reserves. In a non-stressed animal, the glycogen content of the muscle is high. After slaughter, glycogen is converted into lactic acid, which contributes towards meat that is tasteful, tender and of overall good quality. Stress before or during slaughter decreases glycogen content and reduces meat quality [28][29].

During ACTH treatment, cortisol levels where slightly higher than at slaughter but a large variation in the results was noticed. These results confirm the knowledge concerning interindividual differences in behavioural and HPA axis responses observed in cattle [30][31][32]. This variation has also been described by Bertocchi *et al.* (2013) [7] and was explained by the relationship between the circadian rhythm and cortisol excretion in bovine animals.

A significant positive correlation between the concentration of cortisol and that of prednisolone in urine was observed. In case of stress, either at slaughter or pharmacologically-induced increase of cortisol, cortisol levels were elevated and the prevalence of prednisolone was increased, with prednisolone levels ranging from 0.120 to 6.45 μg L^{-1}. The urine sample with the highest prednisolone concentration also showed the highest cortisol level of all collected urine samples (i.e. 368 μg L^{-1}). The European Union Reference Laboratories [33] proposed a regulatory threshold for prednisolone based on the mean of 100 urine samples ± 3 times the standard deviation [13]. Based on their results and previously published data, a threshold level of 5 μg L^{-1} was suggested. Only one urine sample collected during ACTH treatment, demonstrated a urinary prednisolone concentration (i.e. 6.45 μg L^{-1}) above the cited threshold level of 5 μg L^{-1}. No significant differences in prednisolone concentration were observed between the two stress conditions. The prednisolone concentrations found in this study, were in line with Pompa *et al.* (2011) [10], where prednisolone concentrations up to 4.08 μg L^{-1} were observed upon tetracosactide hexaacetate injection in bovines.

An interesting finding of this study relates to the fact that none of the bovines excreted prednisolone, prior to ACTH administration, while all urine samples contained prednisolone after the first administration of ACTH. This can only be explained by assuming that prednisolone is endogenously produced in the animal. Bertocchi *et al.* (2013) [7] showed that a bovine, testing negative for prednisolone at the farm, became positive after transport to the slaughterhouse. Indeed, it has been demonstrated by de Rijke *et al.* (2014) [13], using *in vitro* incubation experiments, that endogenous cortisol and cortisone may be converted by liver enzymes and through faecal bacterial contamination to, respectively, prednisolone and prednisone. Previous studies on the effects of microbial urine contamination already revealed the enzymatic transformation of endogenous testosterone into boldenone through Δ^1-dehydrogenation [35]. A similar reaction was proposed for cortisol and cortisone since both enclose a 3-oxo-4-ene structure, which is a potential target for enzymatic 1,2-dehydrogenation, resulting in the formation of prednisolone and prednisone, respectively [10][36]. Therefore, urine sampling and storage was strictly executed as recommended by De Clercq *et al.* (2013) [15] and particular care was taken to exclude the possible generation of prednisolone due to cross-contamination with faecal or other bacteria [13][35][36], which renders *ex vivo* neoformation of prednisolone due to microbial contamination very unlikely.

Next to determining the prevalence of prednisolone, a main goal of this work was to link the urinary metabolic fingerprint of bovines to the different degrees of stress experienced. Multivariate data analysis indicated that urine samples could indeed be clustered according to the metabolite fingerprints of the newly discovered differentiating metabolites (n = 169), reflecting the imposed stress condition. As such, the metabolic fingerprinting and associated modelling represents a powerful tool to classify an unknown bovine urine sample, offering information about the animal's condition and handling. This is particularly interesting as it may indicate the origin of prednisolone in allegedly non-compliant urine samples.

Multivariate statistics revealed 169 metabolite ions with discriminative value towards the imposed stress conditions. These were screened against an in-house created steroidal database. This revealed that 3 of them were putative steroidal compounds.. Although this number of hits was considered low, it is in line with Gronowska *et al.* (2009) [37] who also reported that major

fluctuations in the urinary steroid profile under influence of stressogenic factors and physiological activity are unlikely.

Eleven ions, which were considered discriminative for the ACTH treatment versus the other conditions, showed a positive correlation with urinary cortisol and/or prednisolone levels. Since it is known that ACTH treatment stimulates the HPA-axis and results in higher urinary cortisol and possibly also prednisolone levels, these 11 ions were linked to either the HPA-axis response during stress or the associated metabolism of cortisol and prednisolone. Using our in-house created database, one of these ions was tentatively identified as 5α-androstan-3(α)(β),17(β)(α)-diol dibenzoate ($C_{33}H_{40}O_4$), which is a compound that has been linked to stress before [38].

Seventeen ions, which were solely excreted during slaughter, could not be correlated to cortisol or prednisolone. This can be explained since stress may affect the adrenal gland in two ways. On the one hand, the short-term stress response at the adrenal medulla causes the hypothalamus to activate the adrenal medulla via nerve impulses. This sympathomedullary pathway results in secretion of epinephrine and norepinephrine [39]. On the other hand, the long-term stress response at the adrenal cortex causes the hypothalamus to activate the adrenal cortex via hormonal signals i.e. CRH and ACTH. This results in secretion of mineralocorticoids and the glucocorticoid cortisol [40][41]. These 17 up and down regulated ions indicated that probably other mechanisms beside the HPA-axis are involved in the stress response. Further research is however warranted to elucidate the identity of these ions as a requisite to determine their relation to stress.

5. Conclusion

In the present study, bovine urinary levels of cortisol, prednisolone and their main metabolites were evaluated under different stress conditions. This revealed that urine of supposedly non-stressed cattle (at the farm) contained no prednisolone. In case of stress, i.e. slaughter and pharmacologically-induced increase of cortisol, cortisol levels were elevated and the prevalence of prednisolone increased. As a consequence, a significant positive correlation between cortisol and prednisolone in bovine urine was demonstrated.

At slaughter, about 60% of the collected urine samples contained prednisolone, whereas after IM tetracosactide hexaacetate treatment prednisolone was detected in all samples. However, no significant difference in prednisolone concentrations could be noticed. Of all prednisolone-positive urine samples, only one showed prednisolone levels above the threshold level of 5 µg L^{-1} i.e. 6.45 µg L^{-1}. This indicates the relevance of the threshold level suggested by the European Reference Laboratories.

After supervised modeling, 169 metabolites were assigned discriminating power towards class separation (i.e. stress condition). For 17 differentiating ions that were upregulated in urine upon slaughter, no correlation with urinary cortisol levels could be noticed. This indicates that besides the HPA-axis, also other processes such as the sympathomedullary pathway are involved in the metabolic urinary pattern during stress. For 11 metabolites upregulated during ACTH treatment, a positive correlation with urinary cortisol and/or prednisolone concentrations was revealed, linking ACTH treatment to the HPA-axis. The specific origin of these ions needs to be further explored since it could give a better insight in the stress response of cattle and the endogenous formation of prednisolone.

Supplementary table 4.1. The 169 differentiating ions with a unique positive (+) or negative (-) contribution during ACTH treatment, at slaughter, or during both. This last is indicated as 'shared'. The order of appearance in the heat map was respected. Ions with positive correlation to urinary cortisol and/or prednisolone concentration levels are indicated in bold.

ID Pos. ions	Measured accurate mass	RT (min)		ID Neg. ions	Measured accurate mass	RT (min)	
1157	442.3315	3.52	Shared +	2015	531.3099	7.69	Shared +
1	432.3461	1.19	Shared +	6470	477.2102	3.24	Shared +
620	428.3155	1.58	Shared +	16056	571.3131	7.85	Shared +
101	430.3315	1.51	Shared +	601	405.1927	6.02	Shared +
1156	430.3314	3.52	Shared +	5205	265.0182	6.79	ACTH +
8	432.3466	1.61	Shared +	13447	164.0019	1.35	ACTH +
1398	432.3470	3.10	Shared +	15592	224.0116	2.84	ACTH +
2803	448.3420	2.67	Shared +	8609	259.0610	4.36	ACTH +
226	430.3314	1.85	Shared +	1020	309.0443	5.73	ACTH +
67	430.3315	2.14	Shared +	**7523**	**203.0178**	**7.36**	**ACTH +**
75	432.3463	1.88	Shared +	9949	266.0687	2.29	ACTH +
142	448.3420	1.97	Shared +	**2363**	**467.1917**	**3.05**	**ACTH +**
2081	462.3208	1.72	Shared +	14005	437.2312	3.67	ACTH +
9928	494.2941	6.07	Shared +	7707	461.1613	7.83	ACTH +
466	430.3313	3.26	Shared +	2313	458.1859	7.80	ACTH +
12	430.3312	2.41	Shared +	3288	473.1796	5.81	SLAUGHTER +
1689	494.2939	5.80	Shared +	9352	511.1595	8.01	SLAUGHTER +
391	448.3412	1.53	Shared +	16589	503.2527	7.97	SLAUGHTER +
525	428.3157	3.14	Shared +	18650	533.3350	8.02	SLAUGHTER +
425	446.3258	2.19	Shared +	12964	349.2608	7.97	SLAUGHTER +
1209	446.3258	1.57	Shared +	15724	491.0522	8.33	Shared -
384	474.3213	3.15	Shared +	9963	293.7551	7.66	Shared -
1882	428.3161	2.84	Shared +	10640	293.7582	7.66	Shared -
90	416.3156	2.32	Shared +	9968	489.0555	8.22	Shared -
342	430.3316	2.70	Shared +	7521	296.7019	7.66	Shared -
5	432.3463	2.22	Shared +	5127	555.5416	7.68	Shared -
3484	578.3818	8.47	Shared +	7114	296.7104	7.66	Shared -
350	446.3266	1.82	Shared +	6887	296.7053	7.66	Shared -
141	446.3258	1.31	Shared +	3268	555.5349	7.66	Shared -
73	302.2146	7.71	Shared +	8969	290.3419	7.69	Shared -
274	428.3160	2.55	Shared +	4509	296.7137	7.66	Shared -
692	430.3315	2.95	Shared +	2747	587.5783	7.66	Shared -
4051	618.9231	8.85	Shared +	18861	261.0448	5.14	Shared -
69	446.3259	1.01	Shared +	4136	296.7171	7.66	Shared -
10692	410.3991	8.87	ACTH +	5486	525.1207	7.60	Shared -
23479	167.0562	8.41	ACTH +	5163	296.7419	7.66	Shared -
29028	230.8931	7.66	ACTH +	2770	587.5709	7.66	Shared -
12555	**219.1490**	**7.26**	**ACTH +**	3866	296.7205	7.66	Shared -
18823	187.0391	2.20	ACTH +	7843	296.7378	7.69	Shared -
19570	302.0573	2.31	ACTH +	4144	296.7341	7.66	Shared -

27340	**182.0754**	**1.58**	**ACTH +**
18137	560.4460	8.25	ACTH +
17229	**509.3836**	**5.94**	**ACTH +**
11539	**293.2221**	**1.54**	**ACTH +**
14184	**491.3730**	**5.94**	**ACTH +**
12324	210.0745	8.14	ACTH +
2211	225.1595	9.04	ACTH +
18743	409.2840	9.28	ACTH +
23248	**501.2944**	**8.79**	**ACTH +**
13108	384.2528	5.19	ACTH +
29815	501.2576	8.47	ACTH +
12206	643.4955	7.72	ACTH +
733	278.2475	2.61	ACTH +
6830	353.1494	1.28	ACTH +
14804	**463.3051**	**2.14**	**ACTH +**
11410	181.0722	7.44	ACTH +
6276	181.0719	4.80	ACTH +
9211	181.0721	6.51	ACTH +
6907	181.0721	4.47	ACTH +
8709	181.0722	5.94	ACTH +
9144	181.0721	6.22	ACTH +
7281	181.0720	5.07	ACTH +
8395	181.0721	5.60	ACTH +
20811	539.3337	8.62	ACTH +
5710	181.0720	3.51	ACTH +
7943	**562.2725**	**8.72**	**ACTH +**
27581	**562.2731**	**8.46**	**ACTH +**
5437	589.3636	2.62	SLAUGHTER +
2877	414.3364	9.28	SLAUGHTER +
18572	474.2867	4.41	SLAUGHTER +
7490	570.3511	3.61	SLAUGHTER +
1955	607.3668	8.17	SLAUGHTER +
17444	290.1783	3.71	SLAUGHTER +
15531	637.3314	7.76	SLAUGHTER +
6277	369.3513	8.55	SLAUGHTER +
8422	428.3156	9.30	SLAUGHTER +
1354	379.3052	4.79	SLAUGHTER +
900	635.3965	8.48	SLAUGHTER +
276	473.3373	7.77	SLAUGHTER +
28541	281.4962	7.71	Shared -
24146	292.9006	7.69	Shared -
26891	281.5191	7.69	Shared -
26604	281.5028	7.69	Shared -
26935	281.5154	7.69	Shared -
25336	281.5125	7.69	Shared -
22806	281.5062	7.69	Shared -
24941	281.5091	7.69	Shared -
11126	349.1185	1.13	Shared -
3578	296.7272	7.66	Shared -
3947	296.7303	7.66	Shared -
17242	356.2568	8.55	ACTH -
2696	367.1075	3.55	ACTH -
8614	293.0495	9.51	SLAUGHTER -
9089	242.9663	5.99	SLAUGHTER -
2372	611.2275	7.92	SLAUGHTER -
254	285.1134	5.78	SLAUGHTER -

9556	271.7227	7.69	Shared -
15114	309.0867	4.80	Shared -
7327	274.3594	7.69	Shared -
2157	485.3000	8.39	Shared -
21850	499.2792	8.40	ACTH -
24859	420.0221	1.11	ACTH -
18169	259.1117	7.70	ACTH -
10086	279.8425	7.73	ACTH -
13236	279.8741	7.73	ACTH -
18437	279.8394	7.70	ACTH -
24544	259.0524	7.69	ACTH -
12350	275.4802	7.67	ACTH -
11955	275.4772	7.72	ACTH -
29338	274.3875	7.72	ACTH -
17934	405.5032	8.47	ACTH -
24581	274.3847	7.69	ACTH -
9764	279.8364	7.72	ACTH -
17690	405.4390	8.49	ACTH -
10438	259.1215	7.73	ACTH -
11681	259.1241	7.73	ACTH -
14500	405.4498	8.49	ACTH -
21416	405.4451	8.48	ACTH -
19989	264.0320	7.69	ACTH -
3627	264.1046	7.67	ACTH -
3494	264.1016	7.72	ACTH -
3407	264.0986	7.72	ACTH -
4418	274.4437	7.67	ACTH -
26802	239.1137	1.04	SLAUGHTER -
20739	255.1373	8.32	SLAUGHTER -
5990	344.1488	8.28	SLAUGHTER -
14092	367.3023	5.96	SLAUGHTER -
25034	233.1511	6.31	SLAUGHTER -
9453	248.1140	1.01	SLAUGHTER -

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CHAPTER V:

PHARMACOKINETIC AND URINARY PROFILING OF GLUCOCORTICOIDS UNDER THERAPEUTIC AND GROWTH-PROMOTING PREDNISOLONE TREATMENT AND PHARMACOLOGICALLY-INDUCED INCREASE OF CORTISOL

Adapted from:

De Clercq, N., Van Meulebroek, L., Vanden Bussche, J., Devreese, M., Fichant, E., Delahaut, P., Croubels, S. and Vanhaecke, L. (2015)

Submitted to *Endocrinology*

Abstract

In Europe, synthetic corticosteroids, such as prednisolone, are not allowed as growth-promoting agents in animal breeding. In the past few years, however, a higher prevalence of non-compliant urine samples for prednisolone without any direct evidence of unauthorized use was reported. Since the urinary prednisolone concentrations after growth-promoting administration are below the threshold suggested by the EURL (5 μg L^{-1}), the use of urinary prednisolone/cortisol concentration ratios and the analysis of 20β-dihydroprednisolone were suggested as potential screening tools to confirm the origin of the urinary prednisolone. To evaluate the applicability and validity of these suggested screening tools, the pharmacokinetic and urinary excretion profiles of prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone were recorded in cattle, subjected to an oral and intramuscular growth-promoting and therapeutic prednisolone treatment, and to pharmacologically-induced increase of cortisol. The latter was induced by administration of tetracosactide hexaacetate, a synthetic analogue of adrenocorticotropic hormone (ACTH). In addition, the metabolism of cortisol was investigated by profiling natural urinary glucocorticoid metabolites during the different treatments. To this extent, bovine urine and plasma samples were collected and analysed using UHPLC coupled to full-scan high-resolution Orbitrap-MS and UHPLC-MS/MS, respectively. Hereby, already 15 min after treatment, 20β-dihydroprednisolone surfaced as the main metabolite of prednisolone in plasma. No significant differences were however noticed between the urinary 20β-dihydroprednisolone levels following growth-promoting prednisolone administration and at 6 h upon ACTH treatment. Determining an appropriate threshold value was hard due to the large variation in data and overlay between endogenous and exogenous concentrations. The prednisolone/cortisol ratios indicated a clear trend during the different treatments, however, further evaluation of ratios obtained in the field remains necessary.

1. Introduction

Synthetic glucocorticoids are extensively employed in cattle for therapeutic purposes because of their well-recognized anti-inflammatory and immunosuppressive properties. Among these glucocorticoids, the most commonly used are dexamethasone, prednisolone, and methylprednisolone. Prednisolone is used in cattle, including dairy cows, for the treatment of allergic dermatitis, otitis, pruritus and musculoskeletal inflammation [1][2]. Apart from the therapeutic applications, synthetic glucocorticoids may also be unauthorized administered to promote the growth of veal calves, finishing bulls and cows at the end of their production cycle [3]. Indeed, glucocorticoids tend to increase live weight gain, improve feed intake, reduce the feed conversion ratio, reduce nitrogen retention, increase the fat content and promote water retention [4]. As such, growth-promoting effects have been demonstrated for beef cattle after oral administration of prednisolone acetate (15 - 30 mg per animal/day) for 30-35 days [5]. Because of the strong pharmacological activity, the residues of most synthetic corticosteroids might impose a risk for food safety. Therefore, to protect consumer's health, the use of synthetic glucocorticoids in livestock is restricted to therapeutic applications only and administration by a licensed veterinarian is requisite [6]. Moreover, appropriate withdrawal times have been defined for glucocorticoid treatment in order to comply with the maximum residue limits (MRLs), established for bovine edible tissues [6][7].

In the past few years, the European Commission reported a higher prevalence of non-compliant urine samples for prednisolone [8][9][10][11] without any direct evidence of unauthorized use. To account for potential endogenous prednisolone, the European Reference Laboratories suggested a threshold level for prednisolone in bovine urine of 5 µg L^{-1} [12][13]. In literature, however, the use of prednisolone/cortisol urinary concentration ratios and the analysis of 20β-dihydroprednisolone were suggested as potential screening tools to confirm the exogenous origin of prednisolone [14][15][16][17], but these have not been confirmed or validated yet. To evaluate the applicability and validity of these suggested screening tools, the pharmacokinetics and urinary excretion profiles of prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone were assessed during both growth-promoting and therapeutic prednisolone treatment (Figure 5.1.).

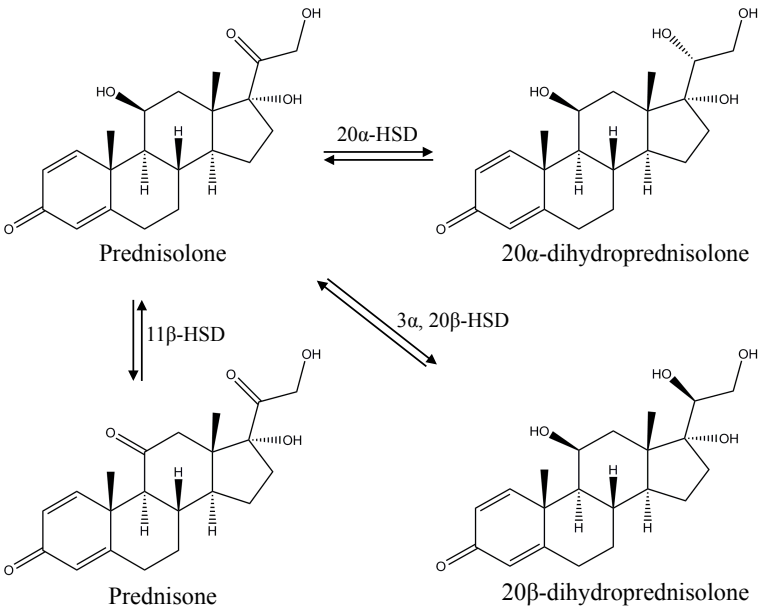

Figure 5.1. Biotransformation pathway of prednisolone into prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone by hydroxysteroid dehydrogenases (HSD).

Recently, the presence of prednisolone was confirmed in bovine urine after stress induction with a synthetic analogue of ACTH, i.e. tetracosactide hexaacetate (Chapter V). Therefore, the pharmacokinetics and urinary excretion of prednisolone and its metabolites during pharmacologically-induced increase of cortisol were considered as well in this study. Only few studies have been devoted to correlate endogenous (e.g. cortisol) and exogenous administered (e.g. dexamethasone, prednisolone) glucocorticoids as a means to estimate the degree of glucocorticoid resistance or supersensitivity [18][19]. Here, the overall cortisol secretion during both growth-promoting and therapeutic prednisolone administrations and during HPA-axis stimulation was assessed, by profiling the urinary natural glucocorticoid metabolites, i.e. cortisone, dihydrocortisone, allotetrahydrocortisol, urocortisol, tetrahydrocortisone, corticosterone, deoxycorticosterone, α-cortolone and 6β-hydroxycortisol.

2. Material and methods

2.1. Animals

In this study twelve clinically healthy cows of a mixed breed were housed under controlled experimental conditions at the animal facilities of Centre d'Economie Rurale (CER, Marloie, Belgium). These cows were 2 to 6 years of age and had a body weight (b.w.) between 370 and 600 kg. They were fed a commercial diet, with *ad libitum* access to water and hay. During the entire study, animals were kept in three separate groups (4 animals per group), all housed in a half covered pen. Prior to the *in vivo* study, an initial acclimatization period of 10 days was foreseen, allowing adaptation to the specific environmental and feeding conditions. This *in vivo* study was approved by CER's Ethical Committee (CE/Sante/ET/004).

2.2. Experimental protocol

After the acclimatization period (Blank), all animals were subjected to a similar oral (*per os*, PO) and intramuscular (IM) prednisolone treatment sequence. First, a growth-promoting treatment (long-term, 40 mg per cow a day, PO and IM) was applied, which was followed by a therapeutic treatment (short-term, 0.5 mg kg^{-1} bodyweight a day, PO and IM) (Figure 5.2.A).

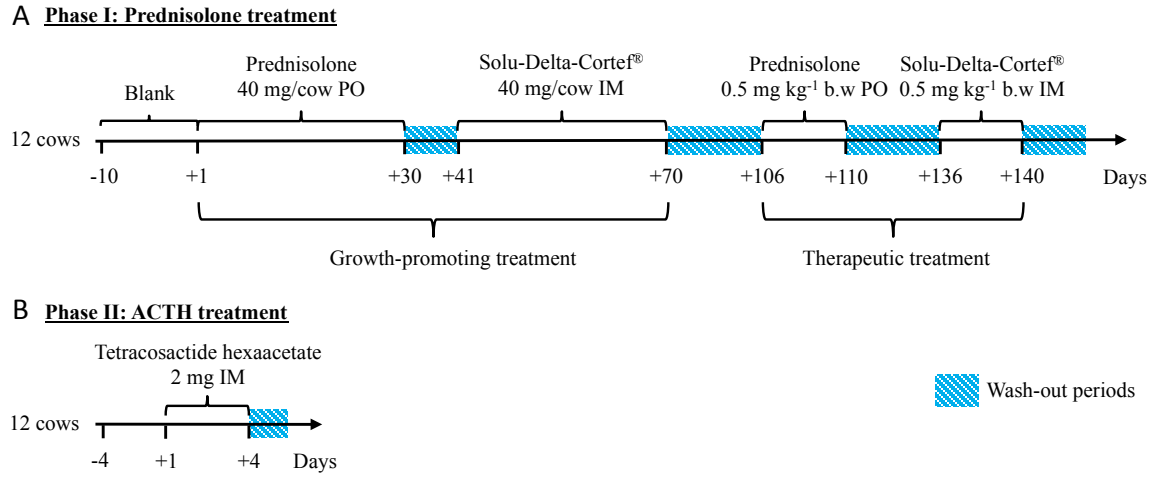

Figure 5.2. Schematic representation of the *in vivo* study, indicating the various experimental sections with oral (PO) and intramuscular (IM) prednisolone administrations (A) and the treatment with tetracosactide hexaacetate, a synthetic analogue of ACTH (B). The wash-out periods are indicated in blue. This experimental procedure was implemented to each animal (n = 12).

Then, a washout period of 11 weeks was incorporated, after which tetracosactide hexaacetate was administered intramuscularly for 4 days to increase cortisol (Figure 5.2.B). All types of administration were executed at 8 a.m.

2.2.1. Phase I: Prednisolone treatment

The growth-promoting treatment started with 30 consecutive days of PO administration of 40 mg day^{-1} of prednisolone in gelulles (prednisolone, Fagron) (Day+1 till Day+30), followed by a washout period of 10 days. Next, IM injections of 40 mg day^{-1} of Solu-Delta-Cortef® (prednisolone sodium succinate, Zoetis, Belgium) were given for 30 consecutive days (Day+41 till Day+70). Before the start of the therapeutic prednisolone treatment, a washout period of 35 days was foreseen.

During the therapeutic prednisolone treatment, a similar experimental set-up was implemented. First, the animals received 0.5 mg kg^{-1} b.w. of prednisolone PO for 5 days (Day+106 till Day+110), which was followed by a washout period of 25 days. Next, IM injections of 0.5 mg kg^{-1} b.w. of Solu-Delta-Cortef® were administered during 5 consecutive days (Day+136 till Day+140). Until 32 days (Day+141 till Day+172) after the last prednisolone administration, urine samples were collected in order to monitor the reconversion to the natural glucocorticoid body state.

During the periods of oral administration, one capsule containing the appropriate amount of prednisolone was given in the morning just after feeding, using a capsule launcher. The IM injections were placed in the neck and were alternated every day from the left to the right neck side in order to reduce irritation.

2.2.2. Phase II: ACTH treatment

After phase I, a washout period of 11 weeks was respected. Subsequently, all animals received IM injections of 2 mg tetracosactide hexaacetate (Utrecht University, Faculty of Veterinary Medicine, Utrecht, The Netherlands), corresponding to 200 I.U. of adrenocorticotropic hormone (ACTH), during 4 consecutive days.

2.3. Sample collection

During the prednisolone treatments, urine samples were collected in the morning, prior to prednisolone treatment. These samples were obtained by a veterinarian using a probe (to prevent faecal contamination) and were immediately portioned into 15-mL tubes, which were then stored in the dark at -80 °C until analysis according to De Clercq *et al.* (2013) [20](Chapter II). During the growth-promoting prednisolone treatment, samples were collected every five days whereas during the therapeutic treatment, urine samples were collected the first, third and last day. In addition, samples were also collected every day during the acclimatization period and every five days during the washout periods. Also, urine samples were collected twice a day during the ACTH treatment period, whereby samples were collected prior to and at 4 h (Day+1 and Day+2) or at 6 h (Day+3 and Day+4) after ACTH administration.

Blood samples were collected in the morning, thereby applying a similar sampling strategy as for urine. In addition, blood samples for pharmacokinetic analysis were collected at the beginning and end of each treatment period at time 0 (just before administration), 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 6 h, and 24 h (post- administration, p.a). With each collection, 5 mL of blood was sampled into heparin tubes. One hour after collection, blood was centrifuged at 600 x g during 15 minutes at 4 °C and divided into 2 mL plasma aliquots. Aliquots were then immediately stored at -20 °C until analysis.

2.4. Reagents and chemicals

Standards of prednisolone, prednisone, cortisone, cortisol, dihydrocortisone, aldosterone, allotetrahydrocortisol, urocortisol, tetrahydrocortisone, corticosterone, deoxycorticosterone, α-cortolone and 6β-hydroxycortisol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Internal standards were cortisol-d_4 (Sigma-Aldrich) and prednisolone-d_8 (TRC, Canada). Reagents were of analytical grade (Merck, Darmstadt, Germany) when used for extraction purposes and of LC-MS Optima grade (Fisher Scientific, Loughborough, UK) for UHPLC-HRMS applications. Ultrapure water was obtained by usage of a purified-water system (VWR International, Merck, Darmstadt, Germany). Primary stock solutions were prepared in ethanol at a concentration of 200 µg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at a range of 0.1 – 10 µg mL^{-1}.

2.5. Sample preparation

2.5.1. Urine

A detailed description of the analytical procedure, which was used for glucocorticoid extraction from urine, has been given in earlier work [20]. In brief, 5 mL of urine was enriched with the internal standards cortisol-d_4 and prednisolone-d_8 to reach final concentration levels of 10 μg L^{-1}. Next, a twofold liquid-liquid extraction with pure tert-butyl methyl ether was applied, whereby the organic phases were collected, pooled and dried under a gentle stream of nitrogen at a temperature of 50 °C. The residue was then dissolved in 100 μL of solvent, corresponding to the initial mobile phase conditions, and transferred into an UHPLC-vial.

2.5.2. Plasma

Two mL of vortexed plasma was spiked with the internal standard cortisol-d_4 to obtain a final concentration of 10 μg L^{-1}. Glucocorticoids were extracted by liquid-liquid extraction, thereby using 5 mL acetonitrile. After 30 minutes of extraction, samples were centrifuged at 3760 x g for 10 min at 10 °C. Then, the supernatants were collected and evaporated under a gentle stream of nitrogen at a temperature of 40 °C. The residue was suspended in 200 μL of water-acetonitrile (80/20, v/v) and transferred into an UHPLC-vial.

2.6. Instrumentation

2.6.1. UHPLC-Orbitrap MS for urine

Glucocorticoid analysis of urine was performed by UHPLC-Orbitrap mass spectrometry, according to the method of De Clercq *et al.* (2013)[20]. Chromatographic separation of the target analytes was thereby achieved using an Accela UHPLC system (Thermo Fisher Scientific, San José, USA), equipped with a Nucleodur Isis C18 column (1.8 μm, 100 mm x 2 mm, Macherey-Nagel, Düren, Germany).

The binary solvent system consisted of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B) (Table 5.1.). High-resolution mass spectrometric analysis was performed with an Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific), equipped with a heated electrospray ionization probe (HESI II), operating in polarity switching mode. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific). This method has been validated according to Commission Decision 2002/657/EC [20][21].

2.6.2. UHPLC-MS/MS analysis for plasma

The chromatographic analysis of glucocorticoids in plasma was performed by a Waters Acquity system (Waters, Manchester, UK) according to Delahaut *et al.* (2013) [16]. Chromatographic separation of the target analytes was thereby achieved using an Acquity C18 column (1.7 μm, 125 mm x 3 mm). The injection volume was 20 μl. The binary solvent system consisted of 0.1% formic acid in water-acetonitrile (78:22, v/v) (A) and 0.1% formic acid in methanol-acetonitrile (78:22, v/v) (B) at a constant flow of 0.6 mL min^{-1}. The gradient started with a solvent mixture (v/v) of 99.2% A and 0.8% B. The percentage of B increased to 5% in the next 4 min, then to 15% in 5 min, and further up to 100% in 0.5 min. This condition was held for 1.5 min. During UHPLC analysis, the column was maintained at 50 °C and the samples at 10 °C. Mass spectrometric analysis was performed with a Waters Xevo TQS tandem mass spectrometer (Waters), operating in the positive ion electrospray mode and applying multiple reaction monitoring (MRM). For each target compound, two transitions were monitored (Table 5.1.), the first being the quantifier and the second, the qualifier. For quantification, two internal standards were used: prednisolone-d_4 and cortisol-d_4. Instrument control and data processing were carried out by MassLynx and QuanLynx software (Waters) respectively.

Table 5.1. MS/MS-parameters for the target glucocorticoid compounds and internal standards

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Prednisolone	361.2	147.0	20	24
		343.0	20	10
Cortisol	363.1	120.8	30	30
		309.0	30	15
Prednisone	359.3	313.3	20	10
		295.0	20	12
Cortisone	361.2	163.0	30	22
		120.9	30	30
Dihydrocortisone	363.2	163.2	36	20
		105.2	36	40
20β-dihydroprednisolone	363.1	267.1	30	15
		171.1	30	20
20α-dihydroprednisolone	363.1	267.1	30	15
		171.1	30	20
Prednisolone-d_4	365.3	347.3	20	10
Cortisol-d_4	367.2	312.2	30	15

A brief validation of the newly developed method for glucocorticoid analysis of plasma was performed based on Commission Decision 2002/657/EC guidelines [21]. The method performance in terms of repeatability, within-laboratory reproducibility, recovery, CC_α and

specificity was thereby assessed. Plasma samples that were used for validation were obtained from non-medicated cows (n = 3), which were housed at the animal facilities of CER. Linearity was evaluated based on eight-point matrix-matched calibration curves with concentration levels ranging from 0.25 to 20 μg L^{-1} for prednisolone, prednisone, 20α-dihydroprednisolone, and 20β-dihydroprednisolone and from 0.5 to 40 μg L^{-1} for cortisol, cortisone, and dihydrocortisone. Repeatability was determined by analysis of samples that were spiked with the target compounds, thereby considering two different concentration levels, i.e. 0.5 and 5 μg L^{-1} for prednisolone, prednisone, 20α-dihydroprednisolone, and 20β-dihydroprednisolone and 1 to 10 μg L^{-1} for cortisol, cortisone, and dihydrocortisone. At each level, the analysis was performed with seven replicates. For evaluation of the within-laboratory reproducibility, the specified analyses were repeated on two different occasions, the second occasion by a different operator. The CC_α was estimated from chromatograms and corresponded to a concentration giving a peak with a signal-to-noise ratio of 3. Specificity was evaluated by analyzing potential interfering substance (methylprednisolone) to identify potential cross-talk during analysis.

2.7. Quantitation and normalization

Due to the broad concentration range expected in the urine samples during the different prednisolone treatments, quantitation of the various urinary glucocorticoid compounds was based on two eight-point calibration curves, which were prepared in urine matrix. Samples were thereby fortified with all glucocorticoid standards to reach concentrations that ranged from 0.50 to 75 ng mL^{-1} and from 100 to 200 ng mL^{-1} for cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, methylprednisolone, 20α-dihydroprednisolone, and 20β-dihydroprednisolone. In this regard, corresponding peak area ratios of the analytes towards the internal standard were used to construct the calibration curves. The employed urine matrix was previously verified to contain no residues of prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone but the other glucocorticoids were found to be endogenously present. Therefore, the endogenous concentration levels of cortisol, cortisone, and dihydrocortisone were determined as the average of five non-fortified urine samples and taken into account during quantitation. In addition, since urine is a matrix prone to dilution,

normalization by means of the specific gravity (Pocket Refractometer™, Atago, Tokyo) was implemented. Calculations were thereby based on the Levine-Fahy equation [22].

2.8. Pharmacokinetic analysis

Pharmacokinetic (PK) analysis was performed with WinNonlin 6.3 (Pharsight Corporation, St-Louis, USA). Plasma concentration-time profiles were modeled using a one- or two-compartmental model for PO and IM prednisolone administration, respectively. The most important PK parameters were calculated comprising the peak plasma concentration (C_{max}), time to reach the peak plasma concentration (T_{max}), area under the plasma concentration-time curve from time 0 to time inf ($AUC_{0\text{-}inf}$), absorption rate constant (k_a), absorption half-life ($T_{1/2a}$), apparent clearance (Cl/F) and apparent volume of distribution (Vd/F). Additionally, for two-compartmental models the distribution rate constant ($k_{el\alpha}$), elimination rate constant ($k_{el\beta}$), elimination half-life ($T_{1/2el\beta}$) were determined. The coefficient of determination was hereby used as an indicator for the goodness-of-fit. For the main metabolites, only C_{max}, T_{max}, $AUC_{0\text{-}inf}$, k_{el} en $T_{1/2el}$ were calculated.

2.9. Statistical analysis

All toxicokinetic parameters from each administered dose were compared between the administration routes using one-way analysis of variance (ANOVA) (p-value ≤ 0.05) (SPSS 21, IBM, USA). The urinary concentrations were statistically evaluated using Student's t-test and one-way ANOVA with post-hoc Tukey's multiple comparisons test.

3. Results and discussion

3.1. Method validation

For each target compound, the correlation coefficient (R^2) was above 0.99 for each of the three calibration curves, established in plasma and analyzed on three different days. The other performance characteristics of the validation are presented in Table 5.2. Recoveries ranged from 92 to 107% (Table 5.2.). Repeatability and within-laboratory reproducibility were evaluated based on the coefficients of variation (RSD) and were below the 15%-tolerance level, specified in CD 2002/657/EC, except for 20α-dihydroprednisolone (22.3% at 0.5 μg L^{-1}), which was nevertheless considered acceptable because of the low target concentration [21].

Table 5.2. Performance characteristics of the method for glucocorticoid analysis in bovine plasma.

Analyte	CC_α (μg L^{-1})	Nominal conc. (μg L^{-1})	Recovery (%)	Precision	
				Repeatability RSD (%)	Within-lab. reprod. RSD (%)
Dihydrocortisone	0.360	1	106.9	13.3	13.1
		10	104.1	3.9	5.6
Cortisol	0.107	1	105.2	13.2	14.3
		10	100.8	3.8	6.3
Cortisone	0.149	1	88.0	9.3	14.7
		10	92.7	6.5	9.5
Prednisolone	0.093	0.5	97.6	4.1	9.9
		5	101.0	3.7	4.4
Prednisone	0.075	0.5	101.6	10.2	13.5
		5	102.7	4.4	12.0
20α-dihydroprednisolone	0.047	0.5	92.7	5.2	22.3
		5	96.4	3.4	12.8
20β-dihydroprednisolone	0.095	0.5	99.1	8.7	14.5
		5	105.4	5.8	10.6

3.2. Pharmacokinetics of prednisolone and its metabolites

The PK parameters were determined for prednisolone during the growth-promoting and therapeutic treatments, thereby using a one- (PO) or two-compartmental (IM) model (Figure 5.3.).

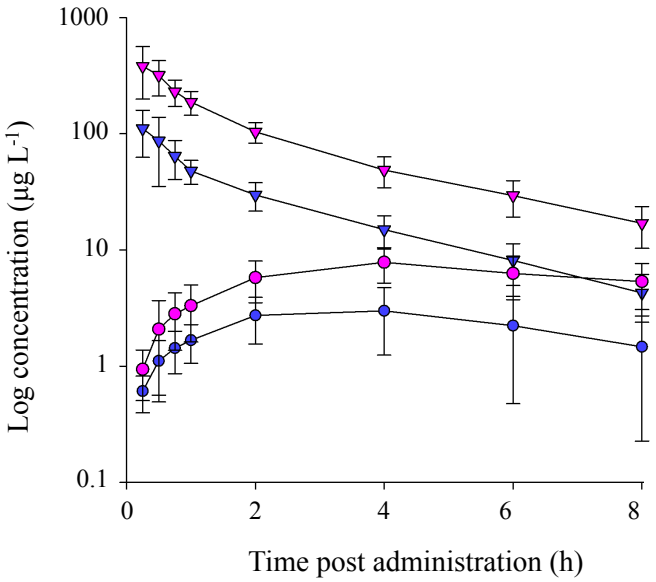

Figure 5.3. Log plasma concentration-time profile of prednisolone during the first 8 h after oral (round) and intramuscular (triangle) administration of prednisolone for growth-promoting (blue) or therapeutic (pink) treatment of cattle (n=12). Results are expressed as mean plasma concentration levels ± SD.

A growth-promoting (40 mg day^{-1}) and therapeutic (0.5 mg kg^{-1} day^{-1}) PO administration of prednisolone to cattle resulted in a relatively fast absorption with a C_{max} at 2.95 h and 3.84 h (T_{max}), respectively. The absorption ($T_{1/2a}$) and elimination half-life ($T_{1/2el}$) for unbound prednisolone after PO and IM administration seemed independent of the dose. However, the highest IM dose (0.5 mg kg^{-1} day^{-1}) had an increased $T_{1/2el}$, which can be explained by saturation of biotransformation in the liver [23]. This saturation explains the significant dose dependency of biotransformation of prednisolone after therapeutic administration. Therefore, therapeutic dosage of prednisolone resulted in significant (p-value ≤ 0.05) higher Cl/F values (Table 5.3.). The Vd/F for oral administered prednisolone increased from 11.95 to 30.06 L kg^{-1} during respectively, growth-promoting and therapeutic treatment. The same effect was noticed during the two types of IM prednisolone administration. This could be explained by nonlinear transcortin binding of prednisolone since this binding is saturated with increased prednisolone concentration [24][25][26].

Table 5.3. Pharmacokinetic parameters (mean ± SD) (n = 12) for prednisolone after oral and intramuscular administration of a growth-promoting (40 mg day^{-1}) and a therapeutic (0.5 mg kg^{-1} day^{-1}) treatment to cattle.

	40 mg day^{-1} PO	**0.5 mg kg^{-1} day^{-1} PO**	**40 mg day^{-1} IM**	**0.5 mg kg^{-1} day^{-1} IM**
C_{max} (µg L^{-1})	2.96 ± 1.50	7.01 ± 1.52	117 ± 25.8	156 ± 78.1
T_{max} (h)	2.95 ± 0.820	3.85 ± 0.909	0.168 ± 0.063	0.866 ± 0.273
$AUC_{0\text{-}tinf}$(h µg L^{-1})	26.1 ± 19.1	75.5 ± 25.0	232 ± 17.5	678 ± 11.5
k_a (h^{-1})	0.363 ± 0.099	0.307 ± 0.129	17.1 ± 11.2	1.93 ± 0.395
$T_{1/2a}$ (h)	2.05 ± 0.588	2.53 ± 0.854	0.064 ± 0.052	0.370 ± 0.083
k_{el} (h^{-1})	0.362 ± 0.091	0.245 ± 0.053	0.325 ± 0.046	0.132 ± 0.092
$T_{1/2el}$ (h)	2.04 ± 0.553	2.97 ± 0.728	2.16 ± 0.308	3.80 ± 0.499
Vd/F (L kg^{-1})	11.9 ± 4.89	30.1 ± 9.98	0.503 ± 0.215	2.47 ± 1.61
Cl/F (L h^{-1} kg^{-1})	4.66 ± 2.68	7.15 ± 1.97	0.346 ± 0.026	0.737 ± 0.012

Pharmacokinetic parameters (one-compartmental) of the main prednisolone metabolites, i.e. prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone, could only be considered for the IM therapeutic prednisolone treatment (Table 5.4), since the plasma concentration levels of all these metabolites were below the associated decision limit CC_α during growth-promoting treatments (PO and IM) and oral therapeutic treatment.

Table 5.4. Pharmacokinetic parameters (mean ± SD) (n = 12) of prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone after intramuscular administration of a therapeutic (0.5 mg kg^{-1} day^{-1}) prednisolone treatment to cattle.

	Prednisone	**20α-dihydroprednisolone**	**20β-dihydroprednisolone**
C_{max} (µg L^{-1})	13.3 ± 2.56	1.64 ± 0.677	19.2 ± 4.31
T_{max} (h)	0.857 ± 0.136	1.42 ± 0.413	2.17 ± 0.571
$AUC_{0\text{-}inf}$ (h µg L^{-1})	58.5 ± 12.1	7.07 ± 3.33	121 ± 26.9
k_{el} (h^{-1})	0.307 ± 0.101	0.553 ± 0.184	0.349 ± 0.086
$T_{1/2el}$ (h)	2.43 ± 0.649	1.53 ± 1.02	2.09 ± 0.522

Based on these results, it was concluded, based on $AUC_{0\text{-}inf}$, that 20β-dihydroprednisolone is the most abundant prednisolone-derived metabolite in plasma. Additionally, 20β-dihydroprednisolone could be detected already 15 min after IM prednisolone administration. Maximum plasma concentration levels for this metabolite were reached about 2 h after start of the treatment (Figure 5.4.).

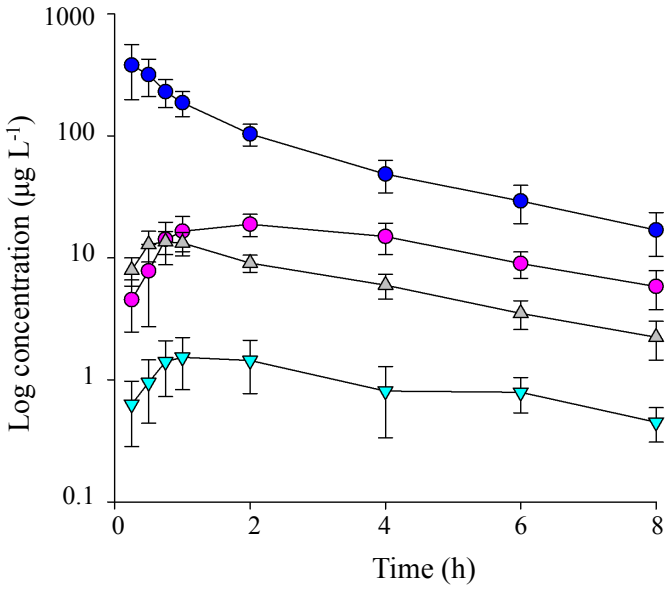

Figure 5.4. Log plasma concentration-time profile of prednisolone (blue) after intramuscular therapeutic administration of prednisolone (0.5 mg kg^{-1} day^{-1}) to cattle (n=12) and formation of the metabolites 20β-dihydroprednisolone (pink), prednisone (grey) and 20α-dihydroprednisolone (turquoise). Results are presented as means ± SD (n = 12).

The T_{max} of prednisolone and prednisone were similar, 0.866 and 0.857 h respectively, which indicates a very fast conversion by the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme in the liver [27].

During tetracosactide hexaacetate administration, traces of prednisolone could be detected in plasma below the CC_{α}.

3.3. Urinary excretion profile of prednisolone and its metabolites

3.3.1. Growth-promoting treatment

Based on the analysis of the urine samples that were collected prior to prednisolone administration, it was verified that prednisolone, prednisone, 20α-dihydroprednisolone, and 20β-dihydroprednisolone were not present at detectable concentration levels. After five days of oral prednisolone treatment (40 mg day^{-1}), urinary prednisolone reached an average concentration of 0.832 μg L^{-1}. However, when animals were given a same dose by IM injection, a mean prednisolone concentration of 1.16 μg L^{-1} was reached (Table 5.5.). These urinary prednisolone concentrations are below the threshold of 5 μg L^{-1} suggested by the EURL. This makes it necessary to determine a potential screening tool to confirm the origin of prednisolone.

During the following 25 days, the urinary prednisolone concentrations remained rather constant for both treatments. Eventually, 24 h after the growth-promoting treatment was ended, prednisolone concentrations started to decrease. Undetectable levels were thereby reached after 5 days, which appeared to be independent of the administration route. The rapid excretion profile of prednisolone was demonstrated earlier by Cannizzo *et al.* (2011) [5], who reported that prednisolone residues were completely eliminated 6 days after a growth-promoting treatment of 35 days. In addition, after PO dexamethasone administration, no residues could be detected in urine after seven days [5][28]. During oral administration of prednisolone, all urine samples contained prednisolone and 20β-dihydroprednisolone, but 20α-dihydroprednisolone was detected in the urine of only 8 out of 12 animals. In none of the urine samples, prednisone could be detected (Table 5.5.).

Table 5.5. Urinary prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone concentrations (μg L^{-1}) during oral (PO) and intramuscular (IM) growth-promoting (40 mg day^{-1}) prednisolone treatment to cattle (n = 12). Means ± SD were determined based on the positive urine samples.

	Prednisolone	**Prednisone**	**20α-dihydroprednisolone**	**20β-dihydroprednisolone**
Growth-promoting PO				
N° positive	12	0	8	12
C_{min} (μg L^{-1})	0.027	-	0.153	0.695
C_{max} (μg L^{-1})	1.17	-	3.41	14.10
Mean (μg L^{-1})	0.832	-	0.771	4.35
SD (μg L^{-1})	0.381	-	0.544	2.44
Growth-promoting IM				
N° positive	12	4	8	12
C_{min} (μg L^{-1})	0.014	0.030	0.138	0.938
C_{max} (μg L^{-1})	8.44	2.17	1.79	14.2
Mean (μg L^{-1})	1.16	0.311	0.472	4.91
SD (μg L^{-1})	1.08	0.413	0.308	3.15

This is in contrast with the urinary excretion profile that was monitored during IM prednisolone administration. Indeed, prednisone was detected in the urine samples from 4 animals. These results are in line with Leporati *et al.* (2013) [17]. In this study it was demonstrated that 20α-dihydroprednisolone, 6β-hydroxyprednisolone and 20β-dihydroprednisone were not present in the urine of PO growth-promoting treated cattle. However the major prednisolone metabolite,

i.e. 20β-dihydroprednisolone, was found until 24 h after the end of the IM treatment, with undetectable levels after five days.

3.3.2. Therapeutic treatment

After three days of PO and IM therapeutic administration of prednisolone, mean urinary prednisolone concentrations of respectively 0.922 μg L^{-1} and 20.1 μg L^{-1} were retrieved. After five days of IM injection, the urinary prednisolone concentration further increased to 42.4 μg L^{-1}, whereas the concentration after PO treatment remained rather constant.

The urinary excretion profile of prednisolone revealed a very strong decrease after ending the PO therapeutic treatment. Within 48h after the final administration, urinary prednisolone concentration levels were diminished with about 80%. Eventually, after 5 days, no more prednisolone or derived metabolites could be detected at concentration levels above the CC_αs. For the IM prednisolone injections, prednisolone levels above the CC_α were detected in two animals up to 10 days after treatment. This is in line with the Nebbia *et al.* (2014) [3], who found urinary prednisolone concentrations above the CC_α in 3 out of 6 animals until 19 days after the last therapeutic treatment, but in contrast with Vincenti *et al.* (2009) [29] where it took 5 days to eliminate and excrete the administered IM therapeutic dexamethasone treatment. In the study of Ferranti *et al.* (2013) [30], no dexamethasone residues could be detected in bovine urine, seven days after a three-day treatment with 2 mg prednisolone.

During oral administration of prednisolone, all urine samples contained prednisolone and 20β-dihydroprednisolone. On the other hand, prednisone and 20α-dihydroprednisolone could only be detected in 2 and 8 animals, respectively (Table 5.6.).

Table 5.6. Urinary prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone concentrations (μg L^{-1}) during oral (PO) and intramuscular (IM) therapeutic (0.5 mg kg^{-1} day^{-1}) prednisolone treatment to cattle (n = 12). Mean ± SD were determined on the number of positive urine samples.

	Prednisolone	Prednisone	20α-dihydroprednisolone	20β-dihydroprednisolone
Therapeutic PO				
N° positive	12	2	8	12
C_{min} (μg L^{-1})	0.038	0.033	0.156	1.07
C_{max} (μg L^{-1})	1.36	0.306	2.85	15.15
Mean (μg L^{-1})	0.922	0.125	0.552	5.13
SD (μg L^{-1})	0.428	0.108	0.635	1.20
Therapeutic IM				
N° positive	12	12	12	12
C_{min} (μg L^{-1})	0.528	1.08	3.32	8.97
C_{max} (μg L^{-1})	189	22.5	127	100
Mean (μg L^{-1})	31.3	8.17	14.9	37.9
SD (μg L^{-1})	40.7	5.91	26.0	25.0

It is remarkable is that 20α-dihydroprednisolone was detected in the same 8 animals as during growth-promoting treatment. This was independent of the body weight but indicated that individual metabolism was responsible for the presence of 20α-dihydroprednisolone in urine after prednisolone administration. This is in contrast with the observations made for the urine samples collected during IM prednisolone administration. Indeed, all of the target glucocorticoids could be detected in the urine along all animals, probably due to the higher bioavailability concentrations reaching the plasma.

At the end of the PO and IM prednisolone treatment, 20β-dihydroprednisolone could be detected in urine until 24 h and 5 days, respectively. The other metabolites were not found after PO administration and only until 24 h after the end of the IM treatment.

For both administration routes, it may be concluded that 20β-dihydroprednisolone is the main known metabolite of prednisolone. This is in agreement with Nebbia *et al.* (2014) [3], who assigned 20β-dihydroprednisolone as the main urinary metabolite of prednisolone in treated animals (n = 14).

3.3.3. ACTH treatment

According to Chapter IV, the synthesis of various glucocorticoids may be induced by treatment with tetracosactide hexaacetate. In this study, detectable concentration levels of prednisolone, prednisone, 20β-dihydroprednisolone and 20α-dihydroprednisolone were observed under this pharmacologically-induced increase of cortisol. Concentration levels for prednisolone in urine ranged from 0.120 to 6.45 μg L^{-1} (average 1.45 μg L^{-1}), at 4 h after tetracosactide hexaacetate treatment (Day+1 and Day+2). After 6 h, prednisolone concentration levels were significantly lower (p-value ≤ 0.05), although still detectable (ranging from 0.169 to 0.729 μg L^{-1}, average 0.318 μg L^{-1}).

The metabolites prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone could also be observed at 4 h after tetracosactide hexaacetate (Day+1 and Day+2) in concentrations ranging from 0.697 to 14.4 μg L^{-1} (average 3.51 μg L^{-1}), 3.31 to 19.1 μg L^{-1} (average 6.31 μg L^{-1}), 0.407 to 33.2 μg L^{-1} (average 8.12 μg L^{-1}), respectively. At 6 h after treatment (Day+3 and Day+4), the metabolite concentrations were 2-3 fold lower, but still detectable in all animals. Eventually, 24 h after the last tetracoasctide hexaacetate administration, no residues of prednisolone or its metabolites could be detected.

In literature, 20β-dihydroprednisolone was suggested as potential biomarker to ascertain unauthorized growth-promoting prednisolone treatment. However, this particular metabolite has been detected in control animals as well [31]. In our results, 20β-dihydroprednisolone was detected upon growth-promoting (PO and IM) prednisolone treatment and after pharmacologically-induced increase of cortisol (Figure 5.5.).

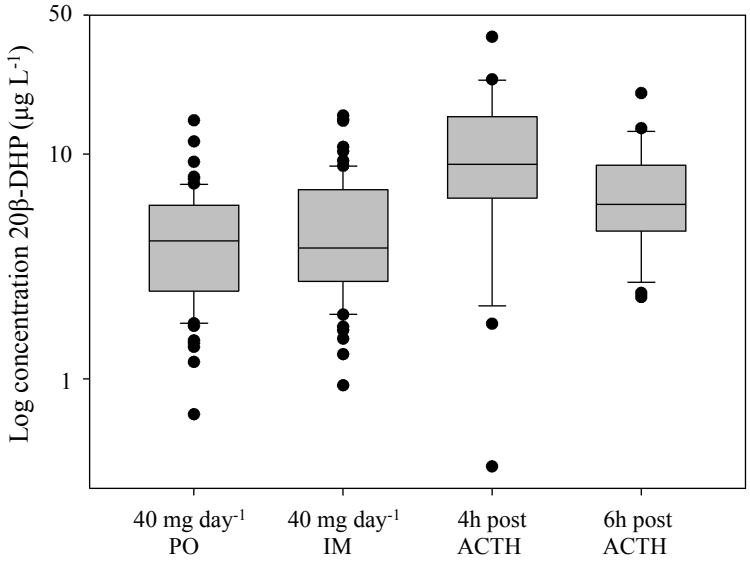

Figure 5.5. Log concentrations of 20β-dihydroprednisolone (20β-DHP) in bovine urine collected during oral and intramuscular growth-promoting prednisolone treatment and during ACTH administration.

No significant difference ($p \geq 0.05$) between the urinary 20β-dihydroprednisolone concentrations after PO and IM prednisolone administration, and at 6 h upon pharmacologically-induced increase of cortisol was noticed. This is in contrast with the urinary concentrations at 4 h post-ACTH treatment, where a significant difference ($p \leq 0.05$) was seen compared to the other time points. However, it is hard to determine a threshold value for 20β-dihydroprednisolone since the urinary concentration quickly decreased when the time between ACTH administration and urine sampling increased. In addition, the large variation among the detected urinary 20β-dihydroprednisolone concentration levels hinders to use this metabolite to discriminate between endogenous and exogenous prednisolone.

3.4. Urinary excretion profile of natural glucocorticoids

3.4.1. Acclimatization

During the acclimatization period, the three most abundant steroids in urine were cortisol (ranging 0.411 to 4.26 µg L^{-1}, average 1.79 µg L^{-1}), cortisone (ranging 0.472 to 5.53 µg L^{-1}, average 2.68 µg L^{-1}) and dihydrocortisone (ranging 0.222 to 9.36 µg L^{-1}, average 2.27 µg L^{-1}). These concentrations are in line with recent *in vivo* studies [15][32][33]. Other steroids and associated metabolites were detected at significantly lower concentrations (Figure 5.6.).

3.4.2. Growth-promoting and therapeutic treatment

The relative intensity changes of the urinary metabolites during the various treatments are visualized by means of a heat map (Figure 5.6.). It was hereby noted that the intensity of most metabolites showed a steady decrease when prednisolone dose increased. Moreover, a significant decrease (p-value ≤ 0.05) of the urinary concentration was observed for cortisol, cortisone, dihydrocortisone, and deoxycorticosterone when a growth-promoting dose of prednisolone was administered, regardless of the administration route. It took 5 days after the end of each growth-promoting treatment, before cortisol and its associated metabolites reached their basal concentration levels again.

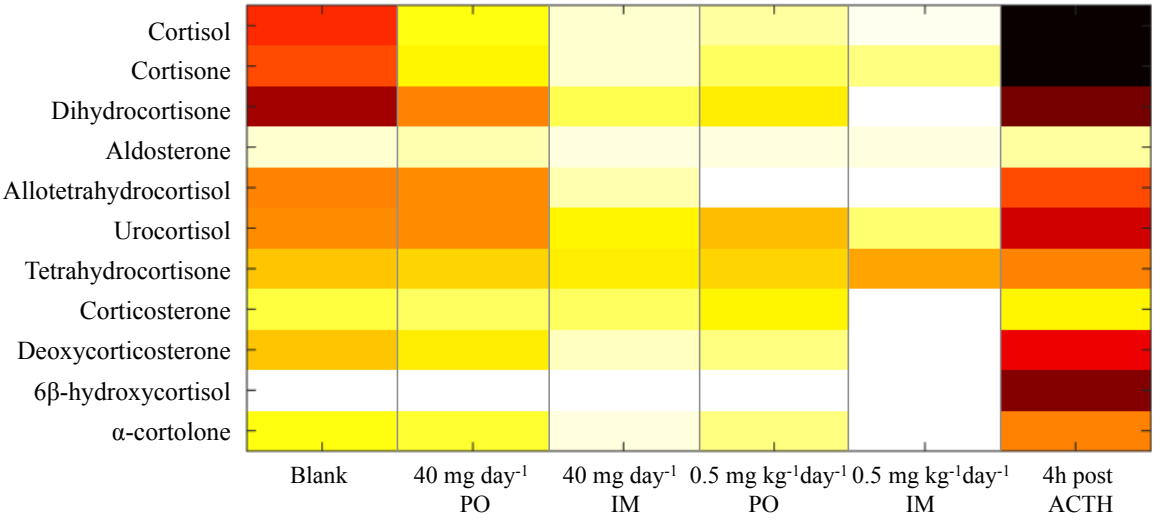

Figure 5.6. Heat map, representing the relative abundance of the selected urinary steroids and metabolites during acclimatization (blank), growth-promoting (PO1 and IM1) and therapeutic (PO2 and IM2) prednisolone treatment, and during administration of tetracosactide hexaacetate. The ion intensities of each ion were averaged and then log transformed. Shades of red and yellow represent higher and lower ion intensities, respectively during the different treatments.

During PO therapeutic administration, a 2- to 5-fold intensity decrease for the urinary metabolites was noticed, while during IM therapeutic treatment, almost no residues of the glucocorticoids dihydrocortisone, allotetrahydrocortisol, corticosterone, deoxycorticosterone, 6β-hydrocycortisol and α-cortolone could be detected. It took 12 days after the end of the therapeutic IM treatment, before cortisol and its metabolites reached their basal concentration levels again.

These findings indicate a significant effect of prednisolone administration on the regulation of the synthesis of endogenous glucocorticoids through suppression of the HPA-axis. This suppression relates to the inhibited CRH transcription by prednisolone and the decreased pro-opiomelanocortin (POMC) gene transcription. Indeed, the associated POMC polypeptide is the precursor of ACTH and its formation is normally stimulated by CRH. As such, through the inhibition of ACTH synthesis, the administration of prednisolone leads to a decreased cortisol production [34]. This negative feedback is influenced by both the dosage and route of administration of prednisolone [23], as was observed in this study as well.

3.4.3. ACTH treatment

Urinary glucocorticoid profiles were evaluated during the first 2 days of ACTH treatment (Figure 5.6.). It was thereby concluded that treatment with tetracosactide hexaacetate significantly affected urinary secretion of cortisol, cortisone, dihydrocortisone, tetrahydrocortisol, 6β-hydrocycortisol and α-cortolone, whereby higher concentration levels were observed (Figure 5.6.). The most intense increases were noticed for cortisol (70-fold), cortisone (40-fold), and 6β-hydrocycortisol (35-fold). Besides, the mineralocorticoid aldosterone was evaluated as well [35], but only a 1.5-fold increase could be noticed which is much less than the observed 70-fold increase of cortisol. Increased cortisol production following ACTH treatment confirmed the potency of this hormone to stimulate the glucocorticoid synthesis by the adrenal gland. These results endorse the findings of Pavlovic *et al.* (2013) [32], whereby 2- to 5-fold increases of cortisol, tetrahydrocortisone and allotetrahydrocortisol were observed under influence of stress at slaughter. The concentrations of cortisol and its associated metabolites 24 h post-treatment were similar to those before tetracosactide hexaacetate treatment. These results are in line with Pompa *et al.* (2011) [36].

The increased cortisol secretion resulted in lower prednisolone/cortisol ratios during ACTH treatment than during growth-promoting prednisolone treatments. In this study, this ratio ranged from 0.0603 to 9.55 during PO growth-promoting prednisolone administration, from 1.57 to 36.9 during IM growth-promoting prednisolone administration, from 0.00379 to 0.0763 at 4 h after ACTH treatment, and from 0.0147 to 0.129 at 6 h after ACTH treatment (Figure 5.7.). A significant difference ($p \leq 0.05$) between the prednisolone/cortisol ratios could be detected,

however, the ratios changed quickly when the time between ACTH administration and urine sampling increased. Therefore, the determination of a valid threshold level for the prednisolone/cortisol level remains hard. Consequently, these results give a first insight in the prednisolone/cortisol ratios under different circumstances (growth-promoting prednisolone administration and artificial induced cortisol levels). However, further evaluation of prednisolone/cortisol ratios in case of natural endogenous prednisolone levels in the field remains necessary.

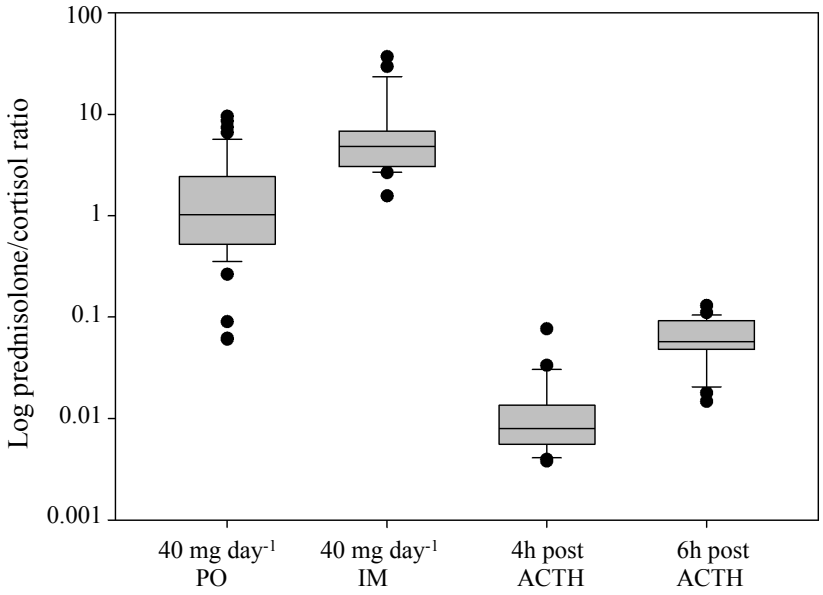

Figure 5.7. Log prednisolone/cortisol urinary concentration ratios in bovine urine collected during oral and intramuscular growth-promoting prednisolone treatment and during ACTH administration.

4. Conclusion

In this study, the pharmacokinetics and urinary excretion profiles of prednisolone and its metabolites were determined, thereby considering different types of prednisolone treatments. Based on these results, it was concluded that 20β-dihydroprednisolone is the main prednisolone-derived metabolite in bovine plasma and urine. Although other metabolites were detected as well, these were only sporadically present in plasma. During ACTH treatment, prednisolone and its metabolites could be detected in urine until 24 h after the end of the treatment. This study confirmed the presence of a negative feedback loop within the HPA-axis

after prednisolone treatment. On the other hand, following ACTH treatment, cortisol secretion increased. Since the urinary prednisolone concentrations after growth-promoting administration were below the threshold suggested by the EURL (5 μg L^{-1}), proper screening tools are necessary to confirm the origin of prednisolone in bovine urine. Therefore, the validity of 20β-dihydroprednisolone and the prednisolone/cortisol ratios as potential screening tools were evaluated. For the metabolite 20β-dihydroprednisolone it was hard to determine an appropriate threshold due to the large variation and overlay between endogenous and exogenous concentrations. The prednisolone/cortisol ratio gave a more clear discrimination, however, further evaluation of ratios obtained in the field remains necessary.

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CHAPTER VI:

METABOLIC FINGERPRINTING REVEALS A NOVEL CANDIDATE BIOMARKER FOR PREDNISOLONE TREATMENT IN CATTLE

Adapted from:

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Abstract

The use of glucocorticoids as growth promoters for meat-producing animals is strictly regulated within the European Union. However, in the past few years, a higher frequency of non-compliant bovine urine samples for prednisolone has been noticed, which could not be directly related to fraudulent use of prednisolone. As such, questions have risen about the origin of this compound. Unfortunately, at present, no decisive strategy has been established to discriminate between endogenous and exogenous prednisolone. In this study, an untargeted metabolomics strategy, based on Orbitrap and QqTOF mass spectrometry, was deployed to reveal urinary biomarkers, which are indicative for the exogenous administration of the synthetic glucocorticoid prednisolone. For this purpose, prednisolone was administered intramuscularly and *per os* to 12 bovines and a total of 2700 urine samples were collected before, during and after treatment. Multivariate statistical data analysis (i.e. OPLS-DA) revealed four differentiating metabolites that allowed discrimination between urine samples collected before and during prednisolone administration. None of these compounds were present in urine containing endogenous prednisolone, of which the formation was induced by the administration of a synthetic analogue of adrenocorticotropic hormone (ACTH). Only one metabolite was retained as a highly suitable biomarker during growth-promoting and therapeutic prednisolone treatment, with 93.4% sensitivity and 96.3% specificity. Besides, this compound could be detected up to four days after a single therapeutic *per os* prednisolone administration. Based on accurate mass, isotope pattern, and MS/MS spectra, this compound was putatively annotated and is suggested as an actionable biomarker for exogenous prednisolone administration.

1. Introduction

Natural glucocorticoids (cortisol and cortisone) are involved in various physiological processes, closely related to immune activity (inflammation). As such, their anti-inflammatory properties have led to the development of synthetic analogs, which proved even more potent. Prolonged exposure to synthetic glucocorticoids, like prednisolone, results in growth-promoting side effects. Therefore, synthetic glucocorticoids may be fraudulently administered to meat-producing animals [1][2]. In order to protect consumers against potential harmful residues, present in animal derived food products, the therapeutic use of synthetic glucocorticoids in livestock has been strictly regulated in the European Union [3]. Maximum residue limits (MRLs) have been introduced for betamethasone, dexamethasone, methylprednisolone and prednisolone in various edible tissues of animal origin [4]. Moreover, the use of synthetic glucocorticoids is completely prohibited for the sole purpose of increasing the body weight of bovines.

In light of the national control plans within the European Union, urine analyses are of critical importance in monitoring illegal administration of glucocorticoids. Recently, the European Commission reported in the Commission Staff Working Document 'Implementation of national residue monitoring plans in the member states' [5][6][7][8] an increasing occurrence of prednisolone residues (3.12 - 179.72 µg L^{-1}) in bovine urine samples without any direct evidence for illegal administration. A number of hypotheses have been suggested to explain this specific finding, i.e. prednisolone could be generated by physiologic metabolic processes under influence of stress, which resulted in higher cortisol levels (during transport and slaughtering) [9][10][11] or by faecal microbial contamination of urine [12]. The latter hypothesis arose from the close structural relationship of prednisolone to cortisol, only differing by one double bond on ring A at the C1-C2 position, which has recently been evidenced by de Rijke *et al.* (2014) [13]. Indeed, *in vitro* incubation experiments of cortisol with bovine liver enzymes showed a significant decrease of cortisol together with formation of prednisolone within six hours. To take into account potential other origins for prednisolone at concentration levels below 5 µg L^{-1}, European Reference Laboratories suggested a threshold level for prednisolone in bovine urine of 5 µg L^{-1} [13][14].

At this time, a method for direct discrimination between endogenous and exogenous prednisolone has not yet been established. One powerful and promising strategy could be the use of isotope ratio mass spectrometry (IRMS). This has already been applied to differentiate synthetic testosterone and estradiol from natural hormones in urine, by considering their inherent and different $^{13}C/^{12}C$ ratios [15][16][17]. However, some significant disadvantages such as low sensitivity and labour-intensive clean-up are associated with this technique [18]. A second promising approach would be an untargeted metabolomics strategy, aiming at the identification of potential biomarkers that allow discrimination between endogenous formation and exogenous administration of prednisolone. This biomarker could, after evaluation, be implemented in the frame of national control plans as screening method [19]. The use of full-scan methods, preferably by means of high-resolution mass spectrometry (HRMS), such as Time-of-Flight (TOF) [20], Fourier Transform Ion Cyclotron Resonance [21] or Fourier Transform Orbitrap MS [22][23], is highly encouraged for such biomarker investigations. Indeed, by screening biological samples with full-scan HRMS, a virtually unlimited number of compounds can be analyzed simultaneously and retrospective post-acquisition evaluation of data can reveal unidentified and/or unknown metabolites [23]. The identification of these metabolites is currently seen as the major bottleneck in the interpretation of metabolomics experiments [24]. In this regard, MS/MS or fragmentation data may enclose valuable identification potential. To obtain these types of data (i.e. both accurate molecular mass and fragmentation pattern) within a single analytical run, hybrid HRMS instruments like Q-Orbitrap-MS and Quadrupole Time-of-Flight-MS are most designated [24][25][26][27]. For further identity confirmation, it is recommended to validate the structure candidates by nuclear magnetic resonance spectroscopy (NMR) [28][29] or by matching the retention time and mass spectra with those of authentic reference standards [30], although these are of course not readily available for every compound [31].

The aim of this work was to reveal potential biomarkers for exogenous prednisolone administration in bovine urine by using a metabolic fingerprinting approach. To this extent, an *in vivo* study was conducted with 12 adult cows that subsequently were subjected to a growth-promoting treatment (low dosage long-term) and a therapeutic treatment (high dosage short-

term) with prednisolone. Collected urine samples were analyzed by both full-scan UHPLC-Orbitrap-MS and UHPLC-QqTOF-MS to acquire the specific metabolic fingerprints corresponding to the different prednisolone treatments. Next, multivariate analysis by means of Orthogonal-Partial Least Squares-Discriminant Analysis (OPLS-DA) was employed to search for differentiating metabolites linked to exogenous prednisolone administration. After metabolite discovery, it is necessary to evaluate the classification performance of the newly defined compounds [32]. The performance of the newly obtained metabolites was defined by sensitivity and specificity [33][34]. In addition, the urinary excretion kinetics of the revealed biomarkers were monitored after a single therapeutic *per os* dose of prednisolone [35]. Insights in these metabolites' kinetics could indeed be of special interest to extend the detection period of illegal prednisolone abuse, i.e. when the detection limit of prednisolone is more rapidly reached than that of the biomarker(s).

2. Material and methods

2.1. Animals

In this study, a diverse group of cattle was compiled in order to include the potential influence of biological variation on the specific biomarker identification. More specifically, twelve healthy cows of a mixed breed, varying age (2 - 6 years), with a body weight between 360 - 570 kg were housed for 8 months under controlled experimental conditions in the animal facilities of the Centre d'Economie Rurale (CER) (Marloie, Belgium). The animals were fed a commercially available diet, commonly applied in zootechnical practice, with *ad libitum* access to water and hay. During the entire study, animals were kept in three separate groups (4 animals per group) and all housed in a half covered pen. Prior to the *in vivo* study, an initial acclimatization period of 14 days was considered. In order to consider animal growth during the *in vivo* study, the animals were weighted at the start and at the end of the experiment, but no significant differences were observed. The *in vivo* study was approved by CER's Ethical Committee (CE/Sante/ET/004).

2.2. Experimental protocol

After the acclimatization period, which served as control, all animals underwent the same sequential prednisolone treatments: a growth-promoting treatment (long-term, 40

mg/cow/day) followed by a therapeutic treatment (short-term, 0.5 mg kg^{-1} b.w./day) (Figure 6.1.). The specific dosages were based on literature findings [10][36][37] to ensure relevant levels of prednisolone and potential metabolites in urine.

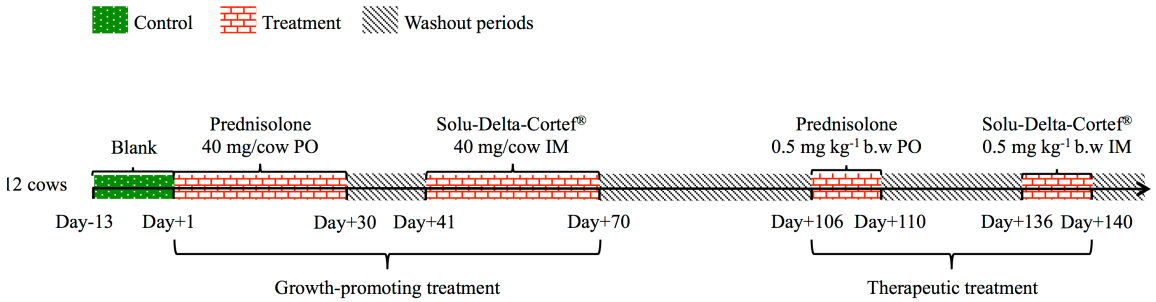


Figure 6.1. Schematic representation of the *in vivo* study, indicating the various experimental sections with oral (PO) and intramuscular (IM) prednisolone administrations and their duration. This experimental protocol was specifically executed to each individual animal (n = 12)

The growth-promoting treatment started with 30 consecutive days of oral administration of 40 mg/day of prednisolone (Sigma-Aldrich St. Louis, USA) (Day+1 till Day+30) (PO1), followed by a washout period of 10 days (WO1). Next, intramuscular injections of 40 mg/day of Solu-Delta-Cortef® (prednisolone sodium succinate, Zoetis, Zaventem, Belgium) were given for 30 consecutive days (Day+41 till Day+70) (IM1). Before the start of the therapeutic prednisolone treatment, a washout period of 35 days was considered (WO2).

During the therapeutic prednisolone treatment, a similar experimental set-up was implemented. First, the animals received 0.5 mg kg^{-1} b.w. of prednisolone *per os* for 5 days (Day+106 till Day+110) (PO2), which was followed by a washout period of 25 days (WO3). Next, intramuscular injections of 0.5 mg kg^{-1} b.w. of Solu-Delta-Cortef® (Zoetis) were administered during 5 consecutive days (Day+136 till Day+140) (IM2). A final washout period of 32 days (Day+141 till Day+172) was respected (WO4).

During the periods of oral administration, one capsule containing the appropriate amount of prednisolone, using lactose as excipient, was given in the morning just after feeding, using a capsule launcher. The intramuscular injections were placed in the neck and were alternated every day from the left to the right side in order to minimize irritation.

2.3. Sample collection

Urine samples were collected in the morning, 5 minutes before prednisolone administration, by a veterinarian using a probe (to prevent faecal contamination), immediately portioned into 15 mL tubes, and stored in the dark at -80 °C until analysis [38]. As for the sampling rate, during the acclimatization period, urine samples were collected daily. During the therapeutic and growth-promoting prednisolone treatments and washout periods, samples were collected every five days.

2.4. Reagents and Chemicals

Standards of prednisolone, prednisone, cortisone, cortisol, 20β-dihydrocortisone and methylprednisolone were purchased from Sigma-Aldrich (St. Louis, USA). Standards of 20α-dihydroprednisolone and 20β-dihydroprednisolone were from Steraloids (Rhode Island, USA). Internal standards were cortisol-d_4 (Sigma-Aldrich) and prednisolone-d_8 (TRC, Canada). Primary stock solutions were prepared in ethanol at a concentration of 200 µg mL^{-1} and stored in dark glass bottles at -20 °C. Working solutions were made in ethanol at a range of 0.1 – 10 µg mL^{-1}.

Reagents were of analytical grade when used for extraction purposes and obtained from VWR International (Merck, Darmstadt, Germany). For UHPLC-HRMS applications, reagents were of LC-MS Optima grade and obtained from Fisher Scientific (Loughborough, UK). Ultrapure water was produced with an Arium 611 UV system (Sartorium Stedim Biotech, Aubagne, France).

2.5. Sample preparation

A detailed description of the analytical procedure for extraction and purification of urine samples has been described by De Clercq *et al.* (2013) [38]. In brief, a five mL aliquot of urine was spiked with internal standards (cortisol-d_4 and prednisolone-d_8) to obtain final concentration levels of 10 µg L^{-1}. Next, a twofold liquid-liquid extraction with pure tert-butyl methylether was performed. The organic phases were collected, pooled and dried under a gentle stream of nitrogen at a temperature of 50 °C. The residue was dissolved in 100 µL solvent, reflecting the initial mobile phase conditions, and transferred to a vial for UHPLC-HRMS analysis.

2.6. Instrumentation

2.6.1. UHPLC-Orbitrap-MS

Untargeted analysis of urine samples was performed by UHPLC-Orbitrap mass spectrometry, according to the validated method of De Clercq *et al.* (2013) [38]. Chromatographic separation was thereby achieved on an Accela UHPLC system (Thermo Fisher Scientific, San José, USA), equipped with a Nucleodur Isis C18 column (1.8 µm, 100 mm x 2 mm, Macherey-Nagel, Düren, Germany). The binary solvent system consisted of 0.1% aqueous formic acid (A) and 0.1% formic acid in acetonitrile (B). The applied gradient program and other chromatographic parameters are presented in Table 6.1. High-resolution mass spectrometric analysis was performed on an Exactive™ single-stage Orbitrap mass spectrometer (Thermo Fisher Scientific, San José, USA), equipped with a heated electrospray ionization probe (HESI-II), operating in the polarity switching mode. Instrument control and data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San José, USA).

2.6.2. UHPLC-QqTime-of-Flight

For structure elucidation of the differentiating metabolites, urine samples were also analysed by UHPLC-QqTOF-MS. This hybrid system enables full-scan HRMS analysis in combination with HR-MRM like fragmentation (MRM^{HR}). The TripleTOF® 4600 mass analyser (SCIEX, California, USA) was coupled to a UHPLC UltraLC 100-XL system (SCIEX), consisting of an Eksigent pumping system, autosampler and degasser. The parameters of the chromatographic separation, using a Nucleodur C18 Isis column (1.8 µm, 100 mm x 2 mm, Macherey-Nagel) (Table 6.1.).

The TripleTOF® 4600 mass analyser was equipped with a DuoSpray™ source, comprising an electrospray (TIS) as well as an atmospheric pressure chemical ionization (APCI) inlet. Ionization of the compounds was achieved using the electrospray inlet (TurboIonSpray® TIS) and operated separately in the positive and negative ion mode. For every 10 samples, automated calibration was performed using an external calibrant delivery system (CDS), which infuses calibration solution prior to sample analysis. The mass spectrometer was operated in the information-dependent acquisition (IDA) mode, combining full-scan TOF-MS survey and MS/MS experiments. During the full-scan survey, spectral data about all ions, present within the selected *m/z* scan range of 150-650 Da, were acquired for each scan. In addition, for every scan the fragmentation

profiles of the top five abundant ions were generated by MS/MS. However, ions that occurred seven consecutive times within the top five of most abundant ions were excluded for MS/MS experiments for 1 sec. Instrument control was carried out by Analyst® TF 1.6 software (SCIEX, California, USA).

Table 6.1. Overview of the specific UHPLC-Orbitrap-MS and UHPLC-QqTOF-MS parameters used during urine analysis.

	UHPLC-Orbitrap-MS		**UHPLC-QqTOF-MS**	
UHPLC				
Column oven temperature	30 °C		40 °C	
Flow	0.30 µL min^{-1}		0.35 µL min^{-1}	
Gradient	*Time (min)*	*B% (v/v)*	*Time (min)*	*B% (v/v)*
	0.0	20	0.0	25
	1.0	25	7.0	25
	6.0	25	8.0	95
	7.0	95	9.0	100
	8.0	100	11.0	100
	10.0	100	11.1	25
	10.1	20	13.0	25
	12.0	20		
Ionization	*HESI (II)*		*DuoSpray™ (TIS)*	
	Spray voltage	4 kV	Ion source gas 1	50 psi[b]
	Sheath gas flow rate	75 au[a]	Ion source gas 2	60 psi
	Auxiliary gas flow rate	7 au	Curtain gas	25 psi
	Sweep gas flow rate	2 au	Temperature	450 °C
	Capillary temperature	280 °C	Ion spray voltage	5500 (-4500) V
	Heater temperature	300 °C		
	Capillary voltage	45 (-32) V		
	Tube lens voltage	95 (-100) V		
	Skimmer voltage	16 (-20) V		
Full-scan MS				
Mass resolution	50,000 FWHM at *m/z* 200		30,000 FWHM at *m/z* 956	
m/z range (Da)	150 to 650		150 to 650	
Maximum injection time	500 ms		200 ms	
MS/MS				
Fragmentation mode	-		IDA	
m/z range	-		50 to 450	
Max. N° of candidate ions	-		5	
Accumulation time	-		150 ms	
Collision Energy	-		30 eV	
Collision Energy Spread	-		15 eV	
Mass resolution	-		25,000 FWHM at *m/z* 195	

2.6.3. ^{1}H-NMR

The NMR spectra of the revealed metabolite biomarker were measured on an Avance II Bruker spectrometer operating at a ^{1}H frequency of 700 MHz and equipped with a 1-mm ^{1}H/^{13}C/^{15}N TXI-z probe to allow maximum sensitivity, taking into account the limited amount of available sample. The dried sample (< 10 μg) was dissolved in ± 10 μL MeOD-d_4 (99.96% D) in order to minimize potential signal interferences from the solvent. All spectra were referenced to the residual solvent-signals at 3.31 (5) ppm for the ^{1}H frequency.

2.7. Chemometric data analysis

In this study, the general workflow of data acquisition and analysis can be organized into multiple steps. A first step relates to the extraction of urine samples. Due to the large number of collected urine samples, sample preparation and analysis were performed in subsequent batches of four animals. The first batch consisted of the urine samples of animals 1, 5, 7 and 12. The second batch contained the urine samples of animals 2, 4, 6 and 11, and the last batch included urine of animals 3, 8, 9 and 10. Each batch was first analyzed on the Exactive™ and later on reanalyzed on the TripleTOF® 4600. Samples were analysed in a random order during each batch. As such, both full-scan data for metabolic fingerprinting and MS/MS fragmentation patterns for identification were acquired. Instrumental stability (quality control measure) during mass spectrometric analyses was verified by considering standard injections. These injections were run at the beginning and repeated every 25 samples. This mixture consisted of cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, methylprednisolone, 20α-dihydroprednisolone and 20β-dihydroprednisolone. The following average coefficients of variance (n = 162) were obtained for the various glucocorticoids: 6.39% for cortisol, 5.51% for cortisone, 6.79% for dihydrocortisone, 3.09% for prednisolone, 4.35% for prednisone, 8.39% for methylprednisolone, 7.61% for 20α-dihydroprednisolone and 6.94% for 20β-dihydroprednisolone. Based on the manuscript of Shah et al. (2000) [39], the acceptance criterion to ensure system stability was set at 15%. Since all absolute and average CVs were well below 15%, appropriate stability during analysis was concluded. In addition, relative retention time changes during analysis were < 1% (i.e. < 0.04 min).

The second step involved data pre-processing (peak list generation), thereby using either Sieve™ 2.1 (Thermo Fisher Scientific, San José, USA) or MarkerView™ 1.2.1.1 (SCIEX, California, USA) software, to respectively process Exactive™ (raw.files) and TripleTOF® 4600 (wiff.files) data files. Taking into account that both full-scan data sets enclosed information about both positively and/or negatively charged ions, it was decided to handle both ionization modes separately during peak list generation [23][40]. Applied parameter values included an *m/z*-range of 150 - 650 Da, a *m/z* width of 5 ppm, a retention time window ranging from 1 to 9.5 min, a peak intensity threshold of 100,000 arbitrary units, a maximum peak width of 0.5 min and a maximum number of 10,000 frames. Furthermore, corrections for inherent chromatographic variability along samples were made during the peak alignment process. This strategy rendered a list of features (detected ions) that were characterized by their *m/z*-value, retention time and signal abundance. The signal intensities of these ions were for each sample normalized by the average signal intensity of the two deuterium labeled internal standards i.e. cortisol-d_4 and prednisolone-d_8, which were supplemented prior to extraction. Moreover, since urine is a matrix subjected to potential dilution effects, a secondary normalization was implemented. As suggested by Jacob et al. (2014) [42], normalization by means of specific gravity (Pocket Refractometer™, Atago, Tokyo) based on the Levine-Fahy equation [42] (correction factor ranged from 1.003 to 1.0044) was implemented.

In the final step, multivariate statistical analysis was performed by means of SIMCA™ 13 software (Umetrics, Malmö, Sweden) in order to reveal significant differences between the metabolic fingerprints, associated with the various treatments. For this particular purpose, OPLS-DA was implemented, for revealing metabolite ions with discriminating power towards the samples' class membership. Prior to this OPLS-DA modelling, data were log-transformed and Pareto scaled ($1/\sqrt{SD}$, where SD is the standard deviation) to induce normality and to standardize the range of independent X-variables, respectively [43]. Within the applied modelling strategy, a qualitative relationship between the X-matrix (detected metabolite ions) and the dependent Y-variable (prednisolone treatment samples 'Treatment' or control urine samples 'Control') was established. In this regard, the Y-variable was defined as a qualitative variable, representing the class membership [44][45][46]. The advantage of OPLS compared to

conventional PLS relates to the applied model rotation whereby class separation is found in the first predictive component (correlated variation) and variation that is not correlated with class separation is found in the orthogonal components (orthogonal variation) [47]. Model-validity was verified by performing a 7-fold internal cross validation-analysis of variance (CV-ANOVA, p-value < 0.01) [48], permutation testing, and considering three model characteristics ($R^2(X)$, $R^2(Y)$ and $Q^2(Y)$). The first model characteristic corresponds to the predictive and orthogonal variation in X that is explained by the model, the second characteristic defines the total sum of variation in Y that is explained by the model and the third refers to the goodness of prediction, calculated by full cross-validation [48][49]. Response permutation testing was performed to estimate the significance of the generated models, whereby the order of elements in the Y-vector was randomly permutated 100 times [49].

2.8. Preliminary biomarker validation

After discovery of differentiating metabolites, it is necessary to evaluate the performance and usefulness of the defined compounds [50] whereby a distinction should be made between analytical method validation and clinical qualification. Validation is defined as the process of assessing the biomarker and its measurement performance characteristics, and determining the range of conditions under which the biomarker will give reproducible and accurate data [51][52]. While clinical qualification is the evidentiary process of linking a biomarker with biological processes and clinical endpoints [52]. Both processes are intertwined since the aim is to link the biomarker with its intended use [53]. In this study, the selectivity, bio-analytical performance, and urinary excretion kinetics of the proposed marker molecules were included as preliminary validation. Statistical analysis was performed using SPSS™ statistics 21.

2.9. Identification of biomarkers

After evaluation of the relevant biomarkers, various identification steps were undertaken, thereby using the TripleTOF® 4600 full-scan MS and MS/MS spectra. In first instance, an *in silico* based strategy was applied. Elemental compositions were thereby determined using Formula Finder, a tool within MasterView™ (SCIEX), and based on precursor mass, fragment masses and isotopic pattern. The elements that were allowed for formula prediction were restricted to the basic elements of natural metabolites, i.e. hydrogen (H), carbon (C), oxygen (O), nitrogen (N) and

sulphur (S). To this extent, a mass deviation window of 5 ppm was allowed. Subsequently, chemical formulas were screened towards an in-house database, which comprises the elemental composition of 1693 steroidal compounds (based on the 11th catalogue edition of steroids from Steraloids inc.). Next, structural elucidation was pursued by applying a combinatorial-based prediction strategy, thereby using the MetFrag software tool [54]. Within this approach, the acquired fragmentation spectrum of an ion is matched towards theoretically predicted fragments of candidate structures from public compound libraries (ChemSpider and PubChem), which allows to rank candidate chemical structures. A second identification strategy was based on compound purification (preparative chromatography) and subsequent ^{1}H-NMR analysis.

3. Results and discussion

3.1. Peak list generation

Peak list generation using Orbitrap-MS data resulted in a metabolic fingerprint, which enclosed 9952 positively charged and 9494 negatively charged ions. However, by excluding the ^{13}C containing ion species, the fingerprint was reduced to only 6637 positively and 5626 negatively charged monoisotopic ions. The same strategy was applied to the QqTOF-MS data and yielded a metabolic fingerprint, which comprised 5085 positive and 8036 negative monoisotopic ion species.

3.2. Predictive modeling

The acquired data matrices were normalized and reorganized into a control group (Control, n = 120) and a treatment group (Treatment, n = 216), which comprised all urine samples that were either collected prior to or during prednisolone treatment. It should hereby be noted that all types of prednisolone administration (*per os* vs. intramuscular) and all different treatment strategies (growth-promoting vs. therapeutic, long- vs. short-term) were combined into the same 'Treatment' group. This was to guarantee that differentiating metabolites were relevant for exogenous prednisolone treatment across administration routes. The multivariate statistical analysis, which aimed to reveal such metabolites, will first be discussed for the data set that originated from the Orbitrap mass spectrometric analysis.

OPLS-DA models were separately constructed for the negatively and positively charged ions (Supplementary Figure 6.1.) and were each time evaluated through various validation strategies, i.e. CV-ANOVA ($p < 0.01$), permutation testing, and three model characteristics ($R^2(X)$, $R^2(Y)$, and $Q^2(Y)$). With respect to the latter strategy, the following parameter values were obtained for the constructed OPLS-DA models (based on either the positive or negative ions, respectively): 0.408, 0.993 and 0.894, and 0.598, 0.977 and 0.782. In this context, good model predictability was assumed if $R^2(Y)$ is ≥ 0.5 [48][55]. As such, based on the various validation strategies, overall good model quality was concluded. Next, to reveal the significance of particular ions to discriminate between the control and treatment class, an S-plot was constructed (Figure 6.2.A).

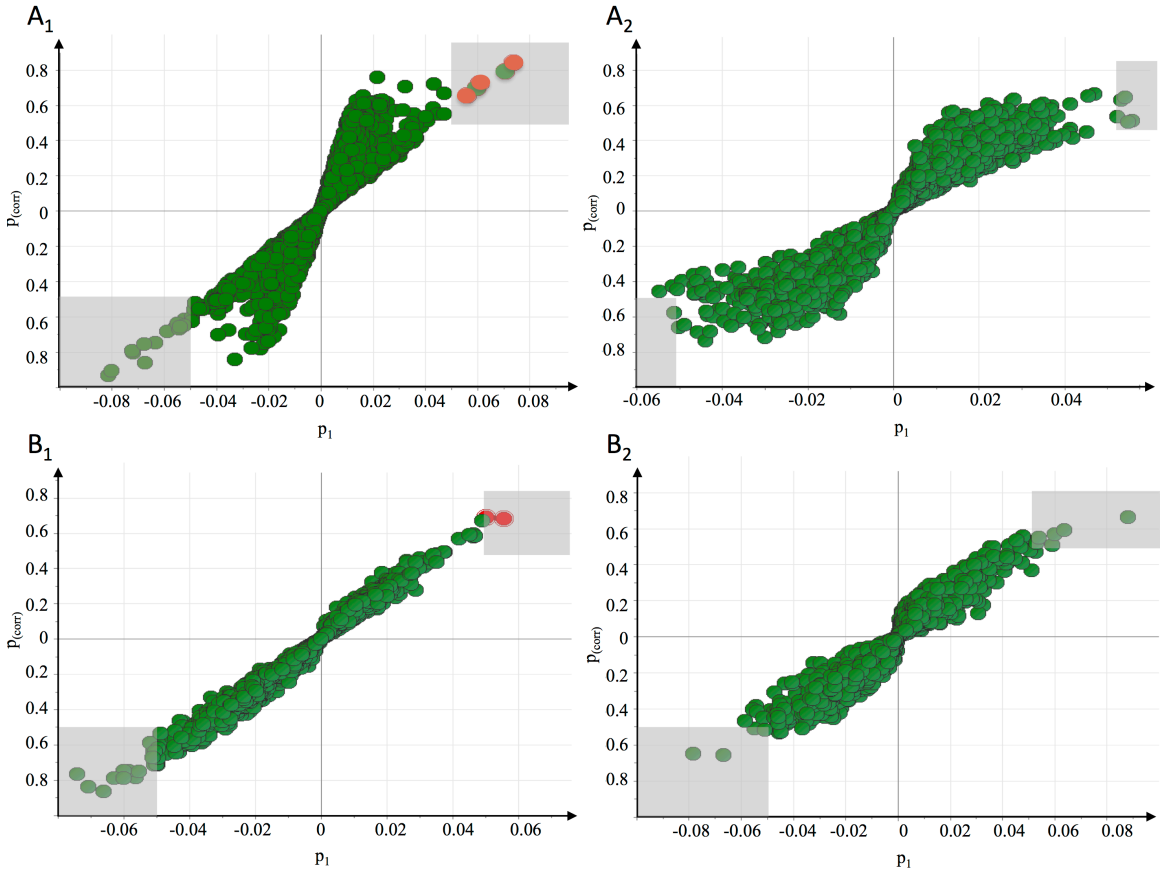


Figure 6.2. Loading S-plot representing the leading contribution of positive (A_1,B_1) and negative (A_2,B_2) ionized metabolite ions, respectively determined with Sieve™ (A) and MarkerView™ (B). Ions with VIP ≥ 3 and $|p_1| \geq 0.05$ and a $|p_{(corr)}| \geq 0.05$ are in red. Depending on the position in the S-plot and the associated p- and p(corr)-values, an ion was significant important towards 'Treatment' (upper, right part of the plot) or towards 'Control' (lower, left part of the plot)

Hereby, the x-axis indicates the contribution (covariance p_1) of a particular ion towards class separation whereas the y-axis refers to the correlation ($p_{(corr)}$) between samples and thus

reliability of results [20][56]. In this regard, ions with cut-off values of $|p_1| \geq 0.05$ and $|p_{(corr)}| \geq 0.05$ were considered as differentiating metabolites [56]. The S-plot results were integrated with the VIP-scores, which indicate the relative importance of the ions to discriminate between classes [57]. In this study, only ions with a VIP Score > 3 were considered as potentially relevant.

As such, 19 positively charged ions and 11 negatively charged ions were eventually retained and considered as differentiating metabolites, allowing to discriminate between endogenous and exogenous prednisolone. In order to determine which of these compounds could serve as an actual biomarker for xenobiotic prednisolone treatment, additional criteria were taken into consideration, i.e. the chromatographic performance (peak shape $A_s \leq 1.5$) [58], signal-to-noise ratio (≥ 3) [59] and points over the peak (≥ 15) [60]. Based on these criteria, 3 positively ionized metabolites were retained and all other metabolite ions excluded. These retained metabolite ions were more strongly detected in the samples that were collected from prednisolone treated animals (Table 6.2.).

Table 6.2. Differentiating metabolites with their respective identification number (ID), retention time (t_R), mass-to-charge ratio (*m/z*), molecular weight (MW), elemental composition of the associated molecule, mass deviation (Δm), Variable Importance in Projection (VIP) score, S-plot score (p_1 and p(corr)).

ID	t_R (min)	*m/z*	MW	Elemental composition	Δm (ppm)	VIP	S-plot	
							p_1	$p_{(corr)}$
Differential analysis with Sieve™								
(1)*	1.41	283.1693	282.1614	$C_{19}H_{22}O_2$	0.1416	6.04	0.073	0.839
2	1.39	265.1589	264.1509	$C_{19}H_{20}O$	0.7919	5.01	0.061	0.723
3	2.00	281.1539	280.1458	$C_{19}H_{20}O_2$	1.0670	4.58	0.056	0.666
Differential analysis with MarkerView™								
(1)*	1.37	283.1699	282.1614	$C_{19}H_{22}O_2$	2.2601	4.10	0.055	0.683
4	2.35	319.1911	318.1825	$C_{19}H_{26}O_4$	2.2400	3.69	0.501	0.689

*: This ion was, independently of the software used for generating the peak lists, by multivariate data analysis assigned as ion with the highest correlation to the exogenous prednisolone treatment.

The same multivariate strategy was applied to the dataset obtained from QqTOF mass analysis. Quality of the established OPLS-DA models was evaluated as described before, considering permutation testing, CV-ANOVA, and three model characteristics. For the constructed models, values for $R^2(X)$, $R^2(Y)$ and $Q^2(Y)$ were 0.371, 0.973 and 0.860 (for the positive ions) and 0.204, 0.877 and 0.665 (for the negative ions). The latter $R^2(X)$ values are rather low, which is explained by the fact that all prednisolone treatment data were combined, resulting in a higher variation. As such, overall good model quality was concluded. Interpretation of the data by means of S-plot

and VIP-scores, thereby using the previously proposed criteria, yielded 16 positively and 10 negatively ion metabolites (Figure 6.2.B). A further exclusion of ions was established by applying the additional peak performance criteria, listed above, whereby only two positively charged ions were retained (Table 6.2.).

Independently of the dataset used for generation of the peak lists, the multivariate data analysis assigned in both cases the same ion (i.e. metabolite biomarker) with the highest correlation to the exogenous prednisolone treatment. This particular ion (further referred to as metabolite 1) was characterized by an *m/z*-value of 283.1693 Da and a retention time ranging from 1.37-1.41 min. In total, four differentiating metabolites were retained.

3.3. Preliminary validation of suggested marker molecules

In literature, numerous metabolites have been claimed as biomarkers for discriminating exogenous treatment with e.g. growth promoters as opposed to natural prevalence. For example, 19-noretiochlanolone [61] and 5α-estrane-3β,17α-diol [62] have been revealed as biomarkers of nortestosterone treatment in porcines and bovines, respectively. However, the differentiating metabolites are in general rarely validated due to the absence of consistent validation guidelines, in essence needed to assign a metabolite the biomarker label [50]. Indeed, although various statistical methods and their limitations are described, no rigorous procedures or criteria are available to evaluate and validate biomarkers, required to endorse their widespread acceptance [63][64][65][66][67]. Upon further inclusion of data from multiple independent validation experiments, the proposal of a threshold as was earlier described by Pinel et al. (2015) [68] and Kaabia et al. (2013) [69] would be recommended. However, setting a threshold based on our preliminary validation data was considered to early. It is generally agreed that a cross-validation experiment, which includes the independent validation of the biomarker by replicating the experiment at different sites, gives high inter-observer and intra-observer reproducibility [50][64][68]. This was unfortunately not possible in light of the experimental complexity of this work. Therefore, a preliminary validation strategy was followed in this study comprising bio-analytical assessment, selectivity evaluation and evaluating urinary excretion kinetics [63][64][65][66][67].

3.3.1. Bioanalytical assessment

Sensitivity and specificity of the four revealed differentiating metabolites are considered of critical importance in gauging their validity as markers for exogenous (*per os* and intramuscular) administration of prednisolone [34][70][71]. Hereby, sensitivity indicates the true positive rate, i.e. the biomarker's presence during treatment, whereas specificity relates to the true negative rate, i.e. the biomarker's absence during control. In this study, sensitivity was determined by assessing the presence of each marker metabolite in the urine samples, obtained during the different prednisolone treatments (Treatment) (Table 6.3.), whereby only metabolites 1 and 4 were assigned a total sensitivity of > 90%. Specificity was determined in the urine samples that were collected during the acclimatization period (Control) and wash-out periods (WO) between the different prednisolone treatments. The first time point of the wash-out periods was excluded due to the unknown elimination kinetics of the differentiating metabolites. Metabolites 1, 2, and 3 were effectively absent in more than 90% of the urine samples, collected outside the prednisolone treatment periods, which indicates a low chance of false positive findings [72].

Table 6.3. Metabolite ions with their respective sensitivity during the different prednisolone treatments, specificity during the acclimatization and washout periods, area under the ROC curve (AUC) and odds ratio.

	ID	1	2	3	4
Sensitivity					
	PO1	100%	100%	91.3%	100%
	IM1	82.6%	72.7%	52.2%	82.6%
	PO2	100%	100%	100%	75.0%
	IM2	100%	85.7%	100%	100%
	Average	93.4%	88.3%	78.7%	90.2%
Specificity					
	Control	100%	100%	100%	86.4%
	WO1	100%	80.0%	87.5%	80.0%
	WO2	96.4%	93.3%	100%	84.8%
	WO3	85.7%	88.9%	88.9%	80.0%
	WO4	92.3%	93.1%	89.6%	82.7%
	Average	96.3%	94.0%	95.5%	84.1%
AUC		1.00	0.94	0.88	0.97
Odds ratio		366	126	79	5

The sensitivity and specificity of each ion were subsequently visualized using receiver-operating characteristic (ROC) curves and its summary index, i.e. the area under curve (AUC) (Table 6.3.).

Normally, the AUC ranges from 0.5 (the area under the diagonal line, representing the extent of class discrimination based on random chance) to 1 (perfect discrimination) [73]. In this study, the AUC obtained for metabolite 1 indicates perfect discrimination, whereas for the other metabolites excellent discrimination could be achieved [32].

The odds ratio [74] was determined to quantify how strong the presence of each biomarker was correlated with prednisolone treatment. In this context, a high odds ratio indicates a strong correlation. The odds ratio of metabolite 1 was much higher than the odds ratios of the other metabolites (Table 6.3.), which evidenced the strong correlation between this biomarker and prednisolone treatment [74].

In conclusion, based on the outlined parameters, metabolite 1 is attributed the highest potential to serve as a reliable biomarker for exogenous prednisolone administration.

3.3.2. Selectivity

To verify that newly defined differentiating metabolites for prednisolone treatment were not present in urine that contained endogenous prednisolone, 12 animals (cfr. Section 2.1) were intramuscularly injected with 2 mg tetracosactide hexaacetate (Utrecht University, Faculty of Veterinary Medicine), a synthetic analogue of adrenocorticotropic hormone (ACTH), corresponding to 200 I.U. of ACTH [9]. After 4 hours, prednisolone was detected in all urine samples, thereby reaching concentration levels that were similar to those obtained during growth-promoting treatments. However, none of the four defined differentiating metabolites were present in these samples, endorsing the ability of these particular metabolites to distinguish between exogenously administered (*per os* and intramuscular) and endogenously synthesized prednisolone [75][76].

3.3.3. Urinary excretion kinetics

By achieving proper insights into the urinary kinetic profiles, the actionable sensitivity in terms of detection window and screening capacities of the proposed biomarkers could be confirmed [35][77][68]. More specifically, an additional *in vivo* experiment was performed in which a single cow (milking cow, 3.5 years, 550 kg body weight) received a single dose of 0.5 mg prednisolone per kg^{-1} b.w. and *per os.* Urine was collected at different time points during the first 32h (4h – 6h30 – 10h – 21h – 24h – 26h30 – 29h30 – 31h15) after administration and alterations in the

peak area of the marker metabolites and prednisolone were monitored. Additional urine samples were collected 4 and 7 days after prednisolone administration (Figure 6.3.). During this experiment, all metabolites were detected, whereby metabolite 1 was five times more abundant (in terms of peak area) in comparison with the other metabolites. Moreover, metabolite 1 was still present at detectable concentration levels (S/N > 3) at the moment that prednisolone was no longer detected in the collected urine samples, i.e. until 4 days after treatment. This specifically endorses the suitability of metabolite 1 as an actionable biomarker for exogenous exogenous prednisolone administration. However, metabolites 2, 3, and 4 may fulfill a supporting role in determining the origin of prednisolone in non-compliant bovine urine samples.

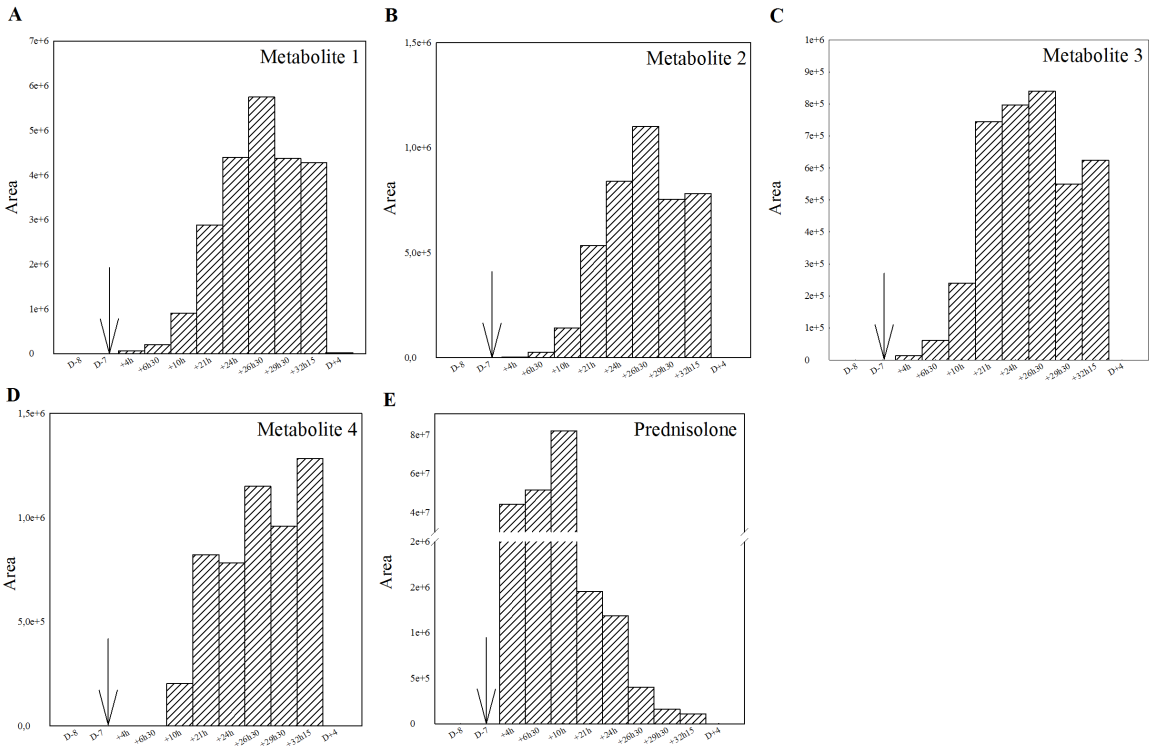


Figure 6.3. Urinary metabolite excretion profile after administration of a single dose of 0.5 mg/kg body weight prednisolone *per os* (indicated with arrow) to one milking cow, with Metabolite 1 (A); Metabolite 2 (B); Metabolite 3 (C); Metabolite 4 (D) and Prednisolone (E). Results are expressed as peak area

3.4. Tentative identification of relevant biomarkers

The elemental composition of the four revealed biomarkers was determined by means of FormulaFinder (MasterView^{TM}), thereby integrating the information about the precursor accurate mass, fragment masses and isotopic patterns. Together with the maximally allowed

mass deviation of 5 ppm, only one candidate chemical formula (Table 6.4.) was suggested for each biomarker and thus used for subsequent structural assessment.

For this particular purpose, the experimental MS/MS spectra from each metabolite ion were matched towards the *in silico* predicted fragmentation pattern from candidate chemical structures, corresponding to the determined chemical formula. These candidate structures were retrieved from publically available databases (i.e. Metlin, PubChem and ChemSpider) and our in-house database. The respective use of MetFrag and MasterView^{TM} software allowed to retain a 'best fitting' candidate structure (Table 6.4.) to the degree of matching fragmentation profiles.

As metabolite 1 was proposed as the most potent biomarker for exogenous prednisolone administration, additional efforts were made to reach the highest level of identification [30]. Since no authentic reference standard was available for the candidate structure, ^{1}H-NMR analysis was attempted to confirm the metabolite's identity. For this reason, all urine samples collected during prednisolone treatments were pooled and preparative HPLC was performed in order to obtain a relatively pure fraction of the compound. Unfortunately, ^{1}H-NMR analysis was not able to reveal the compound's structure, which was mainly due to the available low absolute quantities (< 10 μg) that could be collected for this compound.

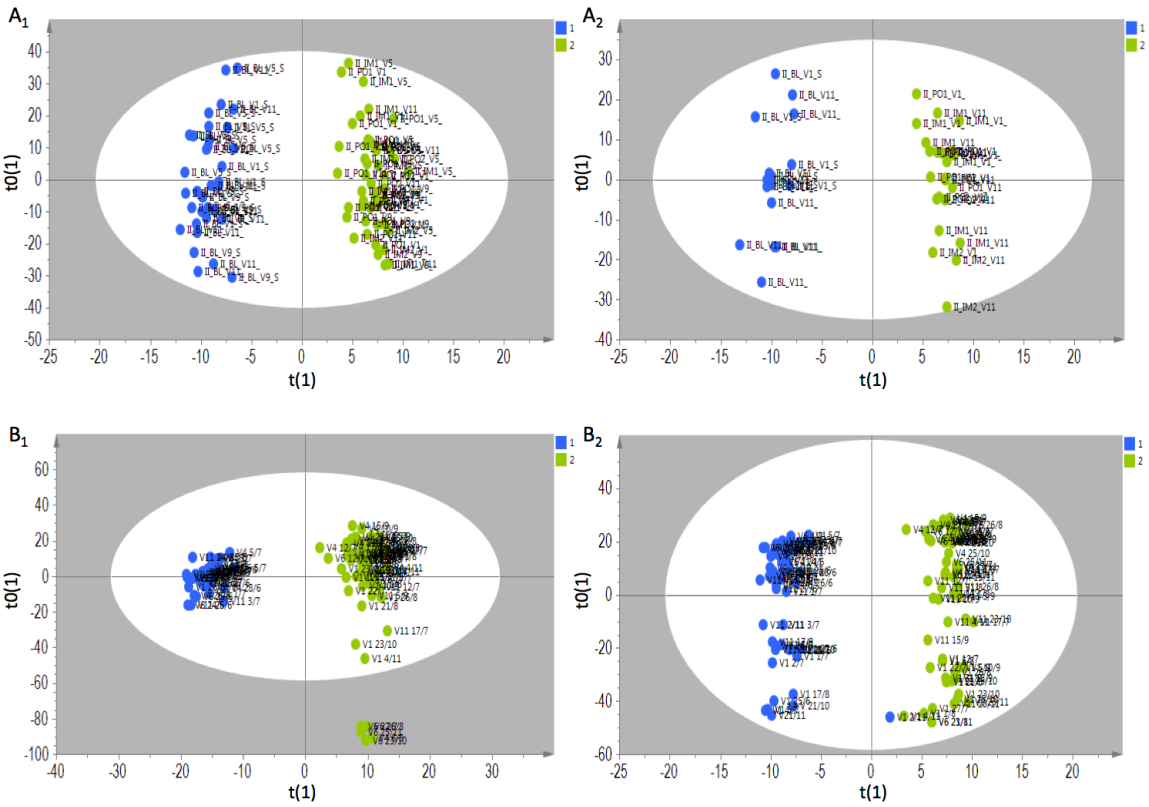
Table 6.4. Metabolite ions with their identification number (ID), the possible identities defined by MetFrag (only the compound with Rank 1 are shown) and the in-house database. The number of matching fragments calculated by MasterView™.

ID	MetFrag			In-house database	
	# Candidate hits	Rank 1	Matching Fragments		Matching Fragments
1	PubChem: 1082	• (E)-2-(tert-butyl)-4-(4-hydroxy-3-methylstyryl)phenol	• 20/63	• 3-methoxy-13-methyl-6,9,11,12,13,14,15,16- • octahydro-17H-cyclopenta[a]phenanthren-17-one	• 1/63
	Chemspider: 473	• 1-[4-(Benzyloxy)phenyl]-5-hexen-1-ol	• 29/63	• 3-methoxy-13-methyl-8,9,11,12,13,14,15,16- • octahydro-17H-cyclopenta[a]phenanthren-17-one	• 1/63
				• 10,13-dimethyl-9,10,11,12,13,14,15,16-octahydro- • 3H-cyclopenta[a]phenanthrene-3,17(8H)-dione	• 1/63
				• 10,13-dimethyl-7,8,10,12,13,14,15,16-octahydro-3H • cyclopenta[a]phenanthrene-3,17(6H)-dione	• 1/63 • 20/63
				• (E)-4-(4-(4-methoxyphenyl)hex-3-en-3-yl)phenol	
2	PubChem: 447	• ((1E,3E)-6-(benzyloxy)hexa-1,3-dien-1-yl)benzene	• 8/94	NF	
	Chemspider: 228	• (E)-1-phenyl-5-(2-vinylphenyl)pent-2-en-1-ol	• 13/94		
3	PubChem: 950	• 2-(7-(methoxymethyl)phenanthren-3-yl)propan-2-ol	• 16/93	• 3-methoxy-13-methyl-11,12,13,14,15,16-hexahydro-	• 1/93
	Chemspider: 480	• Idem		• 17H-cyclopenta[a]phenanthren-17-one	• -
4	PubChem: 648 Chemspider: 483	• 4-(2-{2-[(1S,2R,3R,4R)-3-(Hydroxymethyl)-7-oxabicyclo[2.2.1]hept-2-yl]ethyl}phenyl)butanoic acid	• 17/44	• 5-hydroxy-10,13-dimethyldodecahydro-1H- • cyclopenta[a]phenanthrene-3,6,17(2H)-trione	• 2/44 • -

For now, all revealed biomarkers were putatively annotated (Table 6.4.), thereby reaching the second highest level of identification, as defined by Sumner *et al.* (2007) [30] since a standard for identification at the highest level of confidence is lacking. Besides, the physiological activity and as a result the link to prednisolone administration of the newly defined compounds could not be demonstrated yet, because no literature about these compounds was found. The only retrieved background information comprised the identity of the compounds in online databases Chemspider and/or PubChem.

4. Conclusion

The aim of this study was to identify biomarkers, which have the ability to discriminate between endogenous formation and exogenous administration of the synthetic glucocorticoid prednisolone. A strategy of metabolic fingerprinting was performed to assess potential metabolite perturbations in the urine of cows, treated with prednisolone. Four metabolite ions were found to emerge during prednisolone treatment. The selectivity of the markers was proved, since none of these compounds were present in urine containing endogenous prednisolone, of which the formation was induced by adrenocorticotropic hormone administration. Besides, biological relevance of these ions was determined by means of sensitivity and specificity. This showed that only one metabolite was highly suitable as biomarker during growth-promoting and therapeutic prednisolone treatment, reflected by 93.4% sensitivity and 96.3% specificity. The urinary excretion profiles of the four metabolites were considered as an additional criterion. The most potent compound could be detected up to four days after a single *per os* prednisolone administration. The identity and qualification as fit-for-purpose of the proposed biomarker 'Metabolite 1' needs to be further explored though independent data sets that cover a larger population, different ages, sex, origin of feeding, etc. Moreover, further validation of the proposed biomarker to confirm its specificity for prednisolone treatment as opposed to treatment with other glucocorticoids or growth promoters should be performed.



Supplementary Figure 6.1. Score plots for OPLS-DA models representing the discrimination between control (Blue) and treatment (Green), respectively determined with Sieve™ (A) and MarkerView™ (B) and for positive (A_1,B_1) and negative (A_2, B_2) ionized metabolite ions.

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CHAPTER VII:

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1. Research positioning and relevance

Natural glucocorticoids, with cortisol and cortisone as the main components, are involved in metabolic and immunological responses. In the fifties, synthetic glucocorticoid analogues entered the market because of their greater potency and longer pharmacological activity e.g. dexamethasone, methylprednisolone and prednisolone [1]. Besides their therapeutic use, glucocorticoids are well known for their growth-promoting effects in livestock [2][3]. Illicit treatment may however lead to the presence of potential harmful residues present in animal derived food products [4]. In order to protect consumers against these potential residues, the therapeutic use and the detection thereof has been strictly regulated in the European Union [5][6][7].

Recently, the European Commission reported an increasing prevalence of prednisolone residues in bovine urine samples without any direct evidence for unauthorized administration. These findings may be attributed to an increased sensitivity of the employed analytical detection methods, nevertheless, many questions have been raised about the origin of this prednisolone. One of the hypotheses put forward is that faecal contamination of urine may lead to enzymatic activity such as steroid dehydrogenases, as has been observed for the testosterone to boldenone conversion [8][9]. Due to structural similarities of prednisolone and cortisol, the formation of prednisolone could follow a similar process. Indeed, *in vitro* incubation of cortisol in aqueous faecal solution, led to the formation of prednisolone [10]. This transformation was confirmed in cortisol enriched faecal contaminated urine [11][12]. The influence of microbial contamination on the occurrence of prednisolone required however more research, more specifically regarding the long-term stability of glucocorticoids in urine and faeces. Indeed, a proper conservation protocol could allow detecting, in a reliable fashion, the possible abuse of glucocorticoids excluding *ex-vivo* prednisolone neoformation.

A second hypothesis concerns the relation between stress and the formation of prednisolone, since elevated prednisolone levels in urine samples have been detected at the slaughterhouse [13][14] and following pharmacologically-induced increase of cortisol with tetracosactide hexaacetate, a synthetic analogue of the adrenocorticotropic hormone [15]. But prednisolone

has occasionally been detected under normal housing conditions as well [16]. In this regard, a more in depth evaluation on the influence of stress on prednisolone prevalence in bovine urine was urgently needed.

Besides an elucidation of the mechanisms responsible for prednisolone formation, a direct discrimination between endogenous and exogenous prednisolone could offer potential as well. Also in literature the need for a reliable biomarker has been suggested [3][17], and even more in the light of the national control plans [18]. Recently, the use of the urinary prednisolone/cortisol concentration ratio and 20β-dihydroprednisolone were proposed as potential screening tools for indicating exogenous administration of prednisolone [19], but these had not yet been confirmed or validated. An in depth pharmacokinetic study of prednisolone and its metabolites and their influence on endogenous glucocorticoids could give more insight in the relevance of the suggested screening tools. As long as none of the suggested compounds can be confirmed, the search for specific biomarkers still continues. In this context, untargeted metabolomics by full-scan high-resolution mass spectrometry (e.g. TOF and Orbitrap) offers great opportunities to discover potential biomarkers that may allow discrimination between exogenous and endogenous prednisolone.

2. Main research findings and main scientific contributions

A number of objectives have been defined within the conceptual framework of this thesis and the accomplishments of each have been extensively described in the various research chapters. An overview of the main realisations achieved in this work, is presented in Figure 7.1.

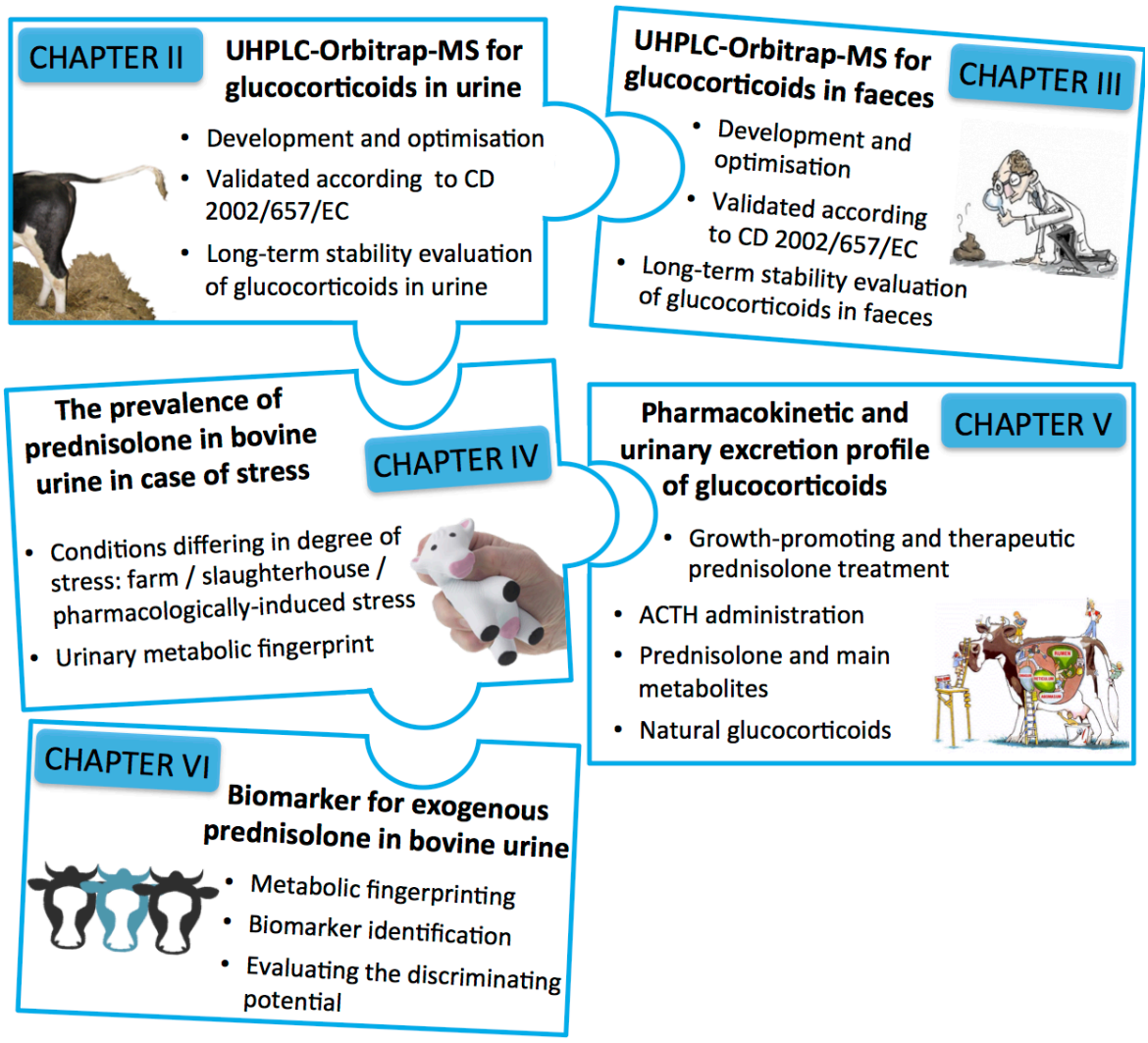

Figure 7.1. Schematic overview of the main accomplishments of this thesis.

2.1. Glucocorticoid extraction and detection with high-resolution Orbitrap-MS

In the context of national residue monitoring plans, urine and faeces are frequently analysed matrices to ensure the absence of glucocorticoid residues in food producing animals [7], since both matrices are easily accessible through non-invasive sampling. The analysis of glucocorticoids in urine and faeces is however seen as a challenging task because they are present at very low concentrations, commingled in a background of abundant primary and secondary metabolites [20]. This endorses the need for proper extraction procedures in order to retain relevant analytes and remove matrix interferences at the same time. But since in this work a metabolomic approach was intended, extraction should be kept as generic as possible.

By using a Plackett-Burman experimental design, sample preparation procedures may be developed in an efficient and time saving manner, enabling identification of the optimal conditions with a minimum of experiments [21][22]. In order to maintain a generic extraction procedure, only a two-step liquid-liquid extraction was implemented for urine while for faeces a defatting step and inclusion of SPE were necessary to reduce matrix interferences co-eluting with the compounds of interest. In order to maintain the generic character of the extraction and to ensure the potential of a full-scan untargeted screening including phase II metabolites, for both urine and faeces, an extraction procedure without hydrolysis was selected.

The development of the analytical methods proved challenging due to the similar chemical configuration and chromatographic behaviour of glucocorticoids. The selection of chromatographic conditions and analytical column were based on literature [23], in-house findings [24] and different experimental setups.

The analytical method for the detection of glucocorticoids and related compounds was based on full-scan high-resolution Exactive^{TM} Orbitrap mass spectrometry. The main relevance of this novel method relates to its ability of post-acquisition re-interrogation of data requisite for the intended metabolomic approach later in this work. After validation of our newly developed UHPLC-HRMS method according to the criteria specified in CD 2002/657/EC [25], it appeared that better or comparable LODs and LOQs were obtained for both urine and faeces [10][11][15][26]. In urine, an experimental determination of the LOQ and LOD was not possible for cortisol, cortisone and dihydrocortisone since no actual blank sample (blank reference

material) was available. Unless solvent would be used for calibration purposes; but this is believed not to be indicative for determining the performance criteria of an analytical method since no interfering matrix compounds are present [27]. For the other glucocorticoids, prednisolone, prednisone and methylprednisolone (not present/detected in the blank urine matrix), blank urine reference material was available and therefore CC_αs and CC_βs could be experimentally determined. In faeces, blank material was available and therefore LOD, LOQ, CC_α and CC_β could be experimentally determined for all analytes. Both methods demonstrated an adequate performance in terms of linearity, repeatability, inter-laboratory reproducibility and precision as well.

These methods were used to determine the long-term stability of glucocorticoids in urine (Chapter II) and faeces (Chapter III). Besides, this full-scan Orbitrap-MS method also allowed glucocorticoid urinary profiling (Chapter IV) and fingerprinting (Chapter III and VI).

2.2. Stability of glucocorticoids in urine and faeces

The collection, transportation and preservation of urine and faecal samples does not always meet ideal conditions. One of the hypotheses for the increased frequency of prednisolone detection in bovine urine samples is the potential transformation of the natural glucocorticoids cortisol and cortisone into prednisolone and prednisone, respectively, due to inappropriate storage and contamination with microorganisms [28][29][30]. Contradictory results have however been reported in literature on this matter [11][28][31]. Therefore, the stability of the potentially endogenous glucocorticoids prednisolone and prednisone and their main metabolites in urine and faeces was considered in this study.

For the experimental setup of the long-term stability study of glucocorticoids in urine and faeces, it was important to pick up sufficient decrease in glucocorticoid levels. To anticipate on this, both matrices were fortified with 20 µg L^{-1}. Based on the LOD/CC_α, this implies that we would be able to detect less then 1.0-2.5% of the initial concentration.

Hereby, multiple conditions were created to evaluate the influence of microbial activity on the possible degradation of glucocorticoids in bovine urine (Chapter II). In case of (anaerobe) faecal contamination, increased prednisolone was observed when preserved at 4 °C. This could

indicate possible neoformation of prednisolone, however, large variability in the data was noticed. After eliminating the bacterial contamination by filter-sterilization, preservation up to 20 weeks at room temperature was possible. But filter-sterilization is a time consuming process and should be performed immediately after sample collection, therefore the pH and preservation temperature were evaluated as well. At extreme pH-values, denaturation of microbial enzymes may occur and prevent glucocorticoid degradation [32]. On the other hand, when stored at -20 °C, preferably at -80 °C, microbial activity is minimized and glucocorticoids remained preserved [28].

Most of the stability studies available for faecal glucocorticoids were performed with antibody assays [31]. The major advantage of monitoring glucocorticoids in bovine faecal material by UHPLC-Orbitrap-MS method (Chapter III) is to eliminate overestimation of the specified glucocorticoids due to cross-reactivity as seen with the more commonly employed antibody assays [33]. Removal of water, by lyophilisation, improved the long-term stability of glucocorticoids in faecal samples. However, lyophilisation is not always possible, therefore alternatives such as addition of ethanol and freezing were considered as well. The addition of ethanol influenced the extraction efficiency. This resulted in unreliable recoveries over time, and is therefore not recommended. Also in literature, the addition of ethanol as conservator for faecal material has given contradictory results [31][34]. Although ethanol immediately stops all bacterial activity within a sample [35], it possibly causes chemical alteration of hormones e.g. oxidation that could influence the outcome of antibody assays [33]. Under anaerobe conditions at room temperature, possible neoformation due to (facultative) anaerobe bacteria (e.g. *E. coli*) was observed. This could strengthen the hypothesis of prednisolone formation due to faecal contamination, however, variation in the data was too big to confirm this. Freezing of bovine faeces at -80 °C, minimized bacterial metabolism and improved stability.

2.3. The influence of stress on the prevalence of prednisolone in bovine urine

In literature it has been suggested that prednisolone can be formed under influence of stress. In response to stress, the hypothalamic-pituitary-adrenal (HPA) axis is stimulated whereby the adrenocorticotropic hormone (ACTH) promotes the synthesis and release of cortisol into plasma, which finally results in increased urinary cortisol levels [36][37][38]. This could be related to the

presence of prednisolone in urine samples, since cortisol only differs from prednisolone by a single ring double bond and this conversion has been demonstrated during *in vitro* experiments [12][30]. The plausibility of this is even strengthened more by the fact that most of the prednisolone positive bovine urine samples were collected at slaughter [13][17]. The influence of stress due to transport to the slaughterhouse has also been demonstrated by Bertocchi *et al.* (2013) [14].

In this study, urine samples collected under three different experimental conditions were tested: supposedly non-stressed animals (farm), animals exposed to 'natural' stress (at slaughter) and animals undergoing pharmacologically-induced increase of cortisol upon administration of tetracosactide hexaacetate, which is a synthetic analogue of ACTH (Chapter IV).

Housing conditions and management procedures at the farm may lead to a potential stressful environment and possibly exert variable effects on the HPA-function in livestock [39]. No significant differences between the urinary cortisol baseline levels at the concerned farms was however noticed. This could indicate the absence of elevated stress levels of animals during their normal housing conditions. None of the urine samples (n = 42) collected under normal housing conditions at four different farms contained prednisolone or prednisone. These results are in line with the large-scale field survey of Vincenti *et al.* (2012)[16].

In case of stress on the other hand, i.e. at slaughter and pharmacologically-induced increase of cortisol, a significant positive correlation between the concentration of cortisol and prednisolone in urine could be observed in our study. Particularly after ACTH treatment, all samples were prednisolone positive. These results are in line with Pompa *et al.* (2011) [15] where similar prednisolone concentrations were consistently found in urine after ACTH treatment. In our study, only one urine sample, collected during ACTH treatment, contained an endogenous prednisolone level higher than the EURL threshold level of 5 µg L^{-1} [18] i.e. 6.45 µg L^{-1}. Based on the findings of our study, a threshold level of 3.5 µg L^{-1} could be proposed. In this case, two urine samples would be misidentified as non-compliant. Hereby the alpha (α = 5%) error for a group B2f compound should be considered, since a false non-compliant decision was made. Besides, it is necessary to take a measurement uncertainty into account, since a recent proficiency test (2012) estimated it at about 50%.

HRMS based metabolic fingerprinting was used to characterise the urinary metabolite patterns under well-defined conditions differing in degree of stress (Chapter IV). PCA-modelling and unsupervised HCA indicated that bovine urinary metabolic fingerprints could be clustered based on their imposed stress situation. This could be a powerful tool to classify unknown bovine urine samples and give information about the stress status of the animal. This could lead to a better interpretation of urine samples tested positive for prednisolone or potentially other conditions related to disturbed hormonal profiles (e.g. Cushing's syndrome) [40][41]. In this context, metabolite ions with a leading role in stress discrimination were defined by means of supervised OPLS-DA (Chapter IV). These ions could be differentiated into three groups: the first group comprised ions predictive for stress but independent of the origin of stress, the second group were ions that were up- or downregulated during ACTH treatment, and the last group were ions that were only retrieved in urine samples collected at slaughter. Correlating the abundance of these ions with cortisol levels was only possible for ions linked to pharmacologically-induced increase of cortisol, indicating that besides the HPA-axis, also other processes are involved in the stress response, e.g. sympathomedullary pathway. This results in the secretion of catecholamines, norepinephrine and epinephrine into the circulation [42]. After degradation into homovanillic acid, normetanephrine, vanillylmandelic acid or metanephrine, these compounds are excreted in urine [43]. Nevertheless, none of these compounds could be directly linked to the metabolite ions that were merely present in urine samples collected at slaughter.

During this study, only urine was considered, although the faecal metabolite fingerprint would reflect the average level of metabolites over a larger time period [44]. Therefore faecal metabolite fingerprint might represent the stress status of an animal more accurately than a single urine sample. But variations in faecal metabolites can only be measured after intestinal passage, which takes 12 to 48 h [45]. This makes faeces irrelevant at slaughter to correlate prednisolone positive samples to transportation stress.

2.4. Pharmacokinetic and urinary profiling of glucocorticoids

Both urinary prednisolone concentrations following growth-promoting administration (Chapter V) and after natural stress and ACTH treatment (Chapter IV) were below the threshold suggested by the EURL (5 µg L^{-1}) [18]. This indicates the need for a proper screening tool to confirm the

origin of the detected prednisolone. In literature, the use of prednisolone/cortisol urinary concentration ratios and the analysis of 20β-dihydroprednisolone were suggested as potential screening tools [16][19][46][47]. In order to evaluate these suggested screening tools, the plasma pharmacokinetic properties and urinary excretion profiles of prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone were assessed by subjecting cattle to a growth-promoting and therapeutic prednisolone treatment (Chapter V). Based on these results, it was concluded that 20β-dihydroprednisolone was the most abundant prednisolone-derived metabolite in plasma and urine. These results are in line with a recent *in vivo* study of Nebbia *et al.* (2014) [4]. However, no significant difference between the urinary 20β-dihydroprednisolone concentrations upon growth-promoting prednisolone administration and 6 h upon pharmacologically-induced increase of cortisol were noticed. Determining an appropriate threshold value was hard due to the large variation in data and overlay between endogenous and exogenous concentrations.

In addition, the metabolism of cortisol was investigated by profiling the natural urinary glucocorticoid metabolites, i.e. cortisone, dihydrocortisone, allotetrahydrocortisol, urocortisol, tetrahydrocortisone, corticosterone, deoxycorticosterone, α-cortolone and 6β-hydroxycortisol. During prednisolone treatment, a steady decrease in relative intensities of these urinary metabolites was noticed. On the other hand, following ACTH treatment, cortisol secretion increased. A significant difference ($p \leq 0.05$) between prednisolone/cortisol ratios could be detected.

Since the urinary prednisolone levels under 'natural' stress (at slaughter) were evaluated in Chapter IV, these prednisolone/cortisol ratios were considered as well in order to establish a suspicious threshold. Hereby the mean value ($\mu_{endogenous}$) of all urine samples with endogenous prednisolone (n = 79) was calculated and added to 2 or 3 times the standard deviation of the same samples ($\mu_{endogenous} + 2\sigma$) or ($\mu_{endogenous} + 3\sigma$) which corresponds to a ratio of 0.299 and 0.426 for the 95^{th} and 99^{th} percentile level of confidence, respectively (Figure 7.2.) [48]. Only six of 149 considered urine samples were misclassified, which indicates that the prednisolone/cortisol ratio has a sensitivity and specificity of 94.2% and 98.7%, respectively. As compared to our newly discovered biomarker (Chapter VI), this is slightly more. Further studies

are warranted to confirm the validity of both as potential biomarker for discriminating endogenous from exogenous prednisolone.

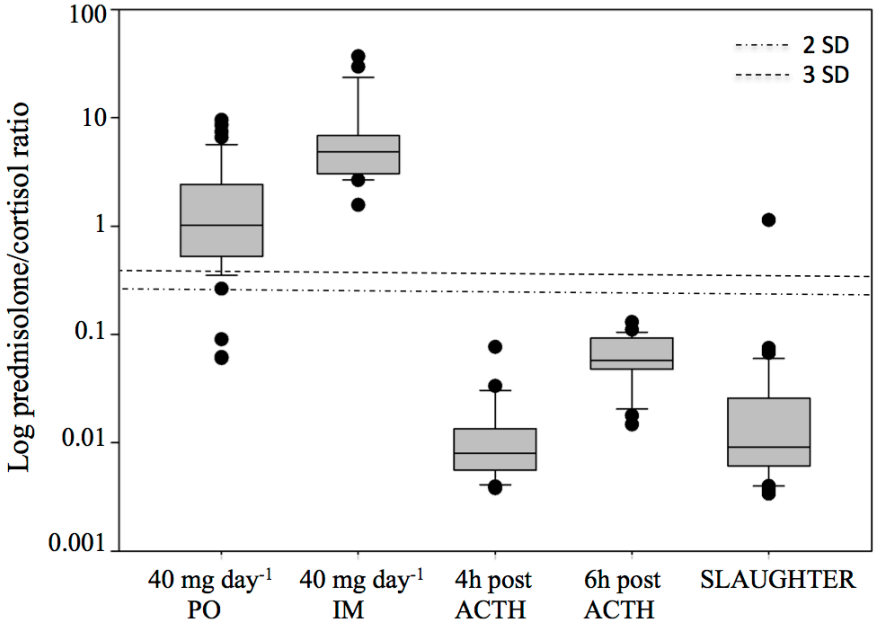

Figure 7.2. Log prednisolone/cortisol urinary concentration ratios in bovine urine collected during oral and intramuscular growth-promoting prednisolone treatment, during ACTH administration and at slaughter.

2.5. A biomarker for exogenous prednisolone treatment in cattle

In literature, the need for a specific biomarker that has the ability to discriminate between endogenous formation and exogenous administration of prednisolone still exists [3][17]. Therefore, an *in vivo* study was conducted in which cattle subsequently were subjected to a growth-promoting and therapeutic treatment with prednisolone (Chapter VI). The specific growth-promoting and therapeutic dosages were based on literature [3][49]. Lower doses were not recommended, since prednisolone and prednisone concentrations retrieved in urine were close to the CC_{α}s (i.e. 1 µg L^{-1} and 0.1 µg L^{-1}, respectively). To ensure relevant levels of prednisolone and potential metabolites in urine, a prednisolone dose of 40 mg day^{-1} was administered during the growth-promoting treatment. Several types of prednisolone treatments (oral *vs.* intramuscular and growth-promoting *vs.* therapeutic) were tested, to guarantee that differentiating metabolites were relevant for exogenous prednisolone treatments across administration routes and dosages. During this metabolomics study, different sources of variation were included in the study subjects (breed, age). Unwanted sources of variation were

minimized i.e. sample collection, preservation, pre-treatment etc. To correct for inter-animal differences, each animal served as its own control.

A strategy of metabolic fingerprinting was followed to discover potential biomarkers in the urine of cows, related to the type of prednisolone treatment. As such, four differentiating metabolite ions emerged independently of the source of variation (oral *vs.* intramuscular, growth-promoting *vs.* therapeutic, age, breed). All markers proved selective, as these were absent in urine containing endogenous prednisolone. The sensitivity and specificity calculations showed that only one metabolite was highly suitable as biomarker during growth-promoting and therapeutic prednisolone treatment showing a sensitivity and specificity of 93.4% and 96.3%, respectively. In addition, the most promising discriminating metabolite was longer detectable in urine than prednisolone i.e. up to four days after a single *per os* prednisolone administration, which emphasizes its potential as a screening tool. If in the light of the national control plan, a suspect bovine urine sample is detected, confirmation with the newly defined biomarker could allow discrimination between endogenous and exogenous prednisolone. To identify discriminating metabolites, this work relied on the fragmentation profiles of Q-ToF. But in order to reach the highest level of identification, additional efforts were made in terms of preparative HPLC and ^{1}H-NMR analysis. Unfortunately, ^{1}H-NMR analysis was not able to reveal the compound's structure, due to the available low absolute quantities (< 10 μg) in the collected urine. For now, all revealed biomarkers were putatively annotated, thereby reaching the second highest level of identification, as defined by Sumner *et al.* (2007) [50] since a standard for identification at the highest level of confidence was lacking.

3. Future perspectives

3.1. Further exploring the underlying mechanisms of prednisolone formation

Several hypotheses were put forward in chapters II, III and IV to explain the 'natural' prevalence of prednisolone in cattle urine, including stress and faecal contamination. Recently, it was also hypothesized that the adrenal origin of prednisolone could not be ruled out [16]. Therefore, it would be interesting to investigate the possible involvement of the adrenal glands by means of an adrenal cell line e.g. H295R. This cell line model facilitates investigations into adrenal steroid hormone production as they can be manipulated to determine cellular responses to external stimuli (e.g. ACTH) [51].

A first insight into the mechanism of cortisol degradation and prednisolone formation was provided during *in vitro* incubation with bovine liver S9 [12]. Nevertheless, additional research to understand the exact biological pathway of prednisolone formation is recommended. To achieve this, several liver-derived *in vitro* models could be used including simple enzyme-containing cellular fractions (i.e. S9 fractions, microsomes) up to whole cell systems (i.e. primary cell cultures, transformed cell lines, tissue slices). The latter are recognized as the closest model to the whole liver in the *in vivo* situation [52]. Combining several types of models will contribute to the subcellular localization of the enzymes involved in the biotransformation [53][54].

3.2. Biomarker identification

During this study, four differentiating urinary metabolites were defined in chapter VI in prednisolone treated cattle by multivariate statistical analysis. Despite the additional efforts made, i.e. ^{1}H-NMR and QqTOF-MS, the compound's structures could not be completely elucidated. Moreover, the suggested structures were not related to the basic steroid skeleton structure. This seems unexpected. However, the urinary fingerprints of the *in vivo* study showed that prednisolone and ACTH treatment affected a wide variety of biological processes (Chapter IV and VI). Therefore it could be anticipated that prednisolone indirectly stimulated the formation of the differentiating metabolites. To investigate the exact biological mechanism(s) that link(s) these markers to exogenous prednisolone, the chemical structure and identity of these compounds and their localization in the organism should be revealed. Therefore, mass

spectrometry imaging (MSI) of biological matrixes (e.g. liver, kidney) could be used. This 2D and 3D surface-based analysis provides deeper knowledge concerning the spatial organization of compounds. During MSI, rigorous sample preparation is essential to achieve the most accurate, reproducible and validated data possible [55][56][57]. In plant sciences, the use of MSI has been demonstrated by detecting a large array of metabolites [58].

3.3. Biomarker validation

Finding differentiating prednisolone/cortisol ratios and metabolites (Chapter VI) is not sufficient to designate them as biomarkers, but more like a first step in the right direction. Before implementation in routine applications, it is necessary to evaluate the performance and usefulness of potential ratios and biomarkers. This process may encounter some difficulties, since the absence of reference guidelines and/or a consensual list of criteria to be fulfilled [59]. This is in contrast with clinical biomarkers where the Food and Drug Agency provided a definition of a valid biomarker [60] and several publications described the way to obtain clinical useful biomarkers [61][62]. Hereby a distinction between analytical method validation and clinical qualification has been made.

During analytical validation, the potency of the analytical method to give reproducible and accurate data of the specific biomarkers is ensured. This depends on the category of the biomarker assay. In our study, the prednisolone/cortisol ratio (Chapter V) is a relative quantitative assay and the stability, linearity, accuracy, precision, sensitivity and specificity should be validated (Table 7.1.). Besides, qualitative biomarkers were defined (present or not present) (Chapter VI). Therefore it is sufficient to show that the analytical method is sensitive and specific enough to detect the targeted analytes. Additionally, the stability of the analytes in a sample should be considered after a freeze/thaw cycle and after leaving the urine samples at the bench top for several hours (Table 7.1.) [61][63][64].

During this study, the newly discovered differentiating metabolites could be determined in every prednisolone treated animal with two kinds of MS techniques i.e. Orbitrap-MS and QqTOF-MS, without noticing interfering compounds. This fulfils the sensitivity and specificity criteria. It should be mentioned that the stability of the biomarkers was not directly evaluated. However, the urine samples collected during the *in vivo* study were preserved at -80 °C as described by De

Clercq *et al.* 2013 [65] (Chapter II) and analysed at different occasions. No significant intensity changes of the defined biomarkers could be noticed. However, the stability of these compounds should be further assessed, thereby focussing on both the effect of multiple freeze/thaw cycles and the exposure to room temperature for several hours.

Table 7.1. Summary of validation parameters applicable to each category of biomarker assay [63].

	Definitive quantitative	**Relative quantitative**	**Quasi-quantitative**	**Qualitative**
Sample stability	X	X	X	X
Reagent stability	X	X		
Assay range	X	X	X	
Parallelism	X	X		
Dilution linearity	X	X		
Accuracy	X	X		
Precision	X	X	X	
Sensitivity	X	X	X	X
Specificity	X	X	X	X

The clinical qualification is the process used to confirm the ratios' and biomarkers' suitability and robustness, in our case discriminating exogenous from endogenous prednisolone. In chapter VI, a first preliminary validation was already performed based on the original dataset [64][65][68]. Of the four newly defined compounds, one revealed high potency as biomarker (*m/z*-value of 283.1693 Da and retention time 1.40 min) during all types of prednisolone treatment. However, profound qualification of the marker is necessary and needs to be performed in an independent dataset i.e. validation dataset. This experiment should cover a larger population in terms of breed (milking cows, meat cows), age, sex, origin of feeding (different farms), animal physiology (different moments of the estrous cycle) and more sampling points following prednisolone treatment (1 to 6 days) [61]. In this way, the differential behaviour of the biomarker can be confirmed and implemented in the frame of national control plans [68][69].

The classical approach (including screening and confirmation) to detect the unauthorized use of prednisolone is not applicable since previous and this work showed that urinary prednisolone could have both an endogenous and exogenous origin [18].

If a suspect bovine urine sample is detected, two approaches could be followed to discriminate endogenous from exogenous prednisolone. The first is based on the EURL suggested threshold

level of 5 µg L^{-1} [18]. However, it must be considered that also lower (i.e. < 5 µg L^{-1}) exogenous prednisolone levels were found during this study. To overcome this problem, screening for our newly discovered biomarker or prednisolone/cortisol ratio could offer an alternative. However, these are currently considered for screening purpose only. The EURL reflection paper states that depending on the significance of the differences observed, biomarkers can be considered as confirmatory methods [18]. However, currently there are no criteria available for this type of analysis. And until now, scientific and statistical considerations are not enough, but full agreement with competent authorities will be needed.

3.4. Other mass spectrometric approaches

During this study, we mainly focussed on the full-scan capacities of the Orbitrap-MS and the measured urinary fingerprints. Another approach to discriminate endogenous from exogenous prednisolone is by considering its isotope ratio with the use of Isotope Ratio Mass Spectrometry (IRMS). This is a versatile application, based on the fact that endogenous compounds reflect an average of all carbon from different origins, while synthetic compounds are elaborated from a single precursor with a defined $^{13}C/^{12}C$ ratio [70][71][72]. Due to the low concentration of the analytes and the small contribution of ^{13}C to the analyte molecule (1%), the use of large volume of samples and an effective clean-up method are required to obtain the minimum of 10 ng of carbon per compound [73]. Numerous methods using GC-combustion-IRMS to distinguish endogenous from synthetic steroids have been published, however, in residue analysis this method is not (yet) routinely used for glucocorticoids.

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SUMMARY

The anti-inflammatory properties of the natural glucocorticoid cortisol have led to the development of synthetic analogues, which exert even higher anti-inflammatory activities. Moreover, these drugs also induce body weight gain in production animals by improving feed intake and lowering feed conversion. Due to their growth-promoting effects, the use of synthetic glucocorticoids is strictly regulated in the European Union (CD 2003/74/EC). Recently, the European Commission reported in the Commission Staff Working Document about the implementation of national residue monitoring plans in the member states (2009 - 2012), an increasing occurrence of prednisolone residues in bovine urine samples without any direct evidence for unauthorized administration. These findings have raised many questions about the origin of this prednisolone. The research of this doctoral thesis intended to further investigate the hypotheses suggested in literature and contribute to the discrimination of endogenous from exogenous prednisolone by using a metabolomic framework.

Chapter I – In this chapter, a comprehensive overview is provided on the origin and chemical configuration of glucocorticoids. In the body, glucocorticoids exert a dual role, which is translated into metabolic and immunological responses. Besides, glucocorticoids are known to display growth-promoting effects, which have led to their abuse in livestock. In this regard, a brief outline of the legislative framework about glucocorticoids is provided. Moreover, a summary is given on the predominant analytical strategies that are used for extraction and detection of glucocorticoids. Herewith, the analytical platform and their capacities that are employed in this doctoral thesis are discussed in detail. As these analytical instruments are able to actualize various metabolomic experiments, the basic principles of metabolomics are defined as well. Finally, the conceptual framework and research objectives of this thesis are formulated.

Chapter II – This chapter describes the development and validation of a generic extraction protocol and full-scan high-resolution mass spectrometry method that enables the analysis of natural and synthetic glucocorticoids in urine. The extraction protocol was optimized by a Plackett-Burman experimental design. Detection of the selected glucocorticoids was achieved by UHPLC-Exactive™ Orbitrap mass spectrometry. This analytical procedure was

validated according to CD 2002/657/EC and included linearity, precision, accuracy, specificity/selectivity and sensitivity. This method was used to examine the stability of glucocorticoids in bovine urine under various storage conditions (up to 20 weeks). This study demonstrated that filter-sterilization of urine, storage at -80 °C, and acidic conditions (pH 3) were optimal for preservation of glucocorticoids and significantly limit degradation up to 20 weeks.

Chapter III – In this chapter an UHPLC hyphenated to Exactive™ Orbitrap-MS method was developed to confirm the presence of glucocorticoids in bovine faeces during a long-term stability study. A Plackett-Burman experimental design was successfully applied to determine the key conditions for optimal extraction of glucocorticoids from faeces. The targeted analysis was successfully validated according to CD 2002/657/EC. The stability study demonstrated that lyophilising faeces and storage at -80 °C were optimal for preservation and significantly limited degradation of glucocorticoids in faeces up to 10 weeks.

Chapter IV – The objective of this chapter relates to the hypothesis that prednisolone could be generated under influence of stress. Therefore, the prevalence of prednisolone in bovine urine under three conditions, differing in degree of stress, was verified: i.e. at the farm, upon slaughter and following administration of a synthetic analogue of the adrenocorticotropic hormone. Hereby, a significant positive correlation between cortisol and prednisolone could be demonstrated. Only one urine sample showed prednisolone levels above the threshold level suggested by the European Reference Laboratories (5 µg/L), which endorsed the relevance of this threshold level. Additionally, the urinary metabolic fingerprint was evaluated and allowed a clear discrimination between the different stress conditions. A total of 169 differentiating metabolites were assigned to have a key role in the urinary pattern in response to stress. This could be a powerful tool to classify unknown urine samples and give information about the stress status of the animals, and may eventually lead to a better interpretation of analysis results of urine samples, which proved non-compliant for prednisolone.

Chapter V – This chapter aimed to evaluate the applicability and validity of urinary prednisolone/cortisol concentration ratios and the analysis of 20β-dihydroprednisolone as potential screening tools to confirm the origin of prednisolone. This by deepening the knowledge on the pharmacokinetic and urinary excretion profiles of prednisolone, prednisone, 20α-dihydroprednisolone and 20β-dihydroprednisolone after oral and intramuscular growth-promoting and therapeutic prednisolone administration, and pharmacologically-induced increase of cortisol in an elaborate *in vivo* study. In addition, the metabolism of cortisol was investigated by profiling the natural urinary glucocorticoid metabolites during the different treatments. A significant negative feedback was noticed during both growth-promoting and therapeutic prednisolone treatments. The metabolite 20β-dihydroprednisolone was not applicable as potential screening tools due to the large variation in data and absence of significant differences during the different treatments. The prednisolone/cortisol ratio was a more promising approach. However, the need for a selective/specific biomarker enabling discrimination between endogenous and exogenous prednisolone remains.

Chapter VI – In this chapter four potential biomarkers were identified that may have the ability to discriminate between endogenously formed and exogenously administrated prednisolone. To this extent, a strategy of metabolic fingerprinting was implemented to assess potential metabolite differences in the urine of cows, subsequently exposed to a growth-promoting and therapeutic prednisolone treatment. After evaluating the sensitivity, specificity, urinary excretion kinetics and selectivity, one compound was found highly suitable as biomarker. Based on accurate mass, isotope patterns and MS/MS spectra, this compound was putatively annotated and may be suggested as an actionable biomarker for exogenous prednisolone administration.

Chapter VII – In this chapter, general conclusions and future research perspectives are formulated. The use of adrenal cell models and liver-derived *in vitro* models were suggested to further explore the underlying mechanisms of endogenous prednisolone formation. In addition, a more profound validation of the biomarker defined in chapter VI should be performed in an independent dataset. After positive evaluation, the biomarker could be

implemented as efficient screening tool to discriminate endogenous from exogenous prednisolone in light of the national control residue plans.

SAMENVATTING

Na de ontdekking van de anti-inflammatoire eigenschappen van natuurlijke glucocorticoïden (cortisol en cortisone) werden talrijke synthetische analogen ontwikkeld met sterkere therapeutische effecten. Daarnaast vertonen deze ook een groeibevorderende werking. Door dit laatste is het gebruik van glucocorticoïden sterk gereglementeerd binnen de Europese Unie (CD 2003/74/EC). De voorbije jaren werden echter in verschillende lidstaten niet-conforme prednisolone resultaten gerapporteerd in runderurine, zonder enige aanwijzing van glucocorticoïdmisbruik. De oorsprong van dit prednisolone is nog niet gekend, hoewel meerdere hypothesen in de literatuur beschreven worden. In deze doctoraatsthesis werd getracht om deze hypothesen nader te bestuderen en een onderscheid tussen endogeen en exogeen prednisolone mogelijk te maken met behulp van een 'metabolomic' strategie.

Hoofdstuk I – In dit hoofdstuk wordt een overzicht gegeven van de oorsprong en chemische configuratie van glucocorticoïden. In het lichaam vervullen glucocorticoïden meerdere functies, wat zich vertaalt in metabole en immunologische reacties. Daarnaast bezitten glucocorticoïden ook groeibevorderende effecten wat voor ongeautoriseerd gebruik zorgde in nutsdieren. Een kort overzicht van de Europese wetgeving wordt hieromtrent gegeven. Vervolgens omsluit dit hoofdstuk een overzicht van de verscheidene analytische technieken, die veelvuldig worden toegepast voor de extractie en detectie van glucocorticoïden. Hierbij wordt vooral ingegaan op het analytische instrumentarium dat gebruikt werd in deze doctoraatsthesis. Aangezien de metabolome screeningsmogelijkheden van de apparatuur hier een belangrijke onderdeel van uitmaken, worden de basisprincipes van metabolomics gedefinieerd. Tenslotte wordt het conceptueel kader geschetst en de opbouw van de verschillende onderzoeksfasen aangegeven.

Hoofdstuk II – Dit hoofdstuk beschrijft de ontwikkeling en validatie van een holistisch extractieprotocol en analytische methode voor natuurlijke en synthetische glucocorticoïden in runderurine. Het extractieprotocol werd geoptimalizeerd met behulp van een Plackett-Burman experimenteel design. Scheiding en detectie van de geselecteerde glucocorticoïden werd uitgevoerd met een UHPLC gekoppeld aan hoge-resolutie Exactive^{TM} Orbitrap massaspectrometrie. Bovendien werd via validatie vastgesteld dat de ontwikkelde methode een correcte kwantificatie van glucocorticoïden toelaat. Deze validatie werd uitgevoerd volgens CD 2002/657/EC waarbij lineariteit, precisie, juistheid, gevoeligheid en specificiteit/selectiviteit

werden geëvalueerd. Deze analytische methode werd toegepast om de stabiliteit van glucocorticoïden in runderurine, onder verschillende omstandigheden gedurende 20 weken, na te gaan. Hierbij werd vastgesteld dat glucocorticoïden in urine best stabiel blijven in aangezuurde urine (pH 3) bij een temperatuur van -80 °C en vrij van bacteriën door middel van filtersterilisatie.

Hoofdstuk III – In dit hoofdstuk werd een UHPLC gekoppeld aan Exactive^{TM} Orbitrap-MS methode ontwikkeld om de aanwezigheid van glucocorticoïden in runderfeces tijdens een lange termijn stabiliteitsstudie na te gaan. Met behulp van een Plackett-Burman experimenteel design werden de optimale extractie omstandigheden voor glucocorticoïden in feces bepaald. Deze analytische methode werd succesvol gevalideerd volgens de richtlijnen beschreven in CD 2002/657/EC. In een lange termijn stabiliteitsstudie werd aangetoond dat vriesdrogen en onmiddellijke bewaring bij -80 °C de beste manier is om de degradatie van glucocorticoïden te minimaliseren gedurende 10 weken.

Hoofdstuk IV – Dit hoofdstuk onderzoekt de hypothese die de mogelijke vorming van prednisolone beschrijft onder invloed van stress. Hiervoor werd de prevalentie van prednisolone nagegaan onder drie verschillende omstandigheden, gekarakteriseerd door een verschillend niveau aan stress: op de boerderij, aan de slachtlijn en na toediening van een synthetisch analoog van het adrenocorticotroop hormoon dat stress induceert. Hierbij werd een significante positieve correlatie tussen cortisol en prednisolone waargenomen. Uit de resultaten blijkt dat slechts één urinestaal een hogere prednisoloneconcentratie bevat dan de grenswaarde voorgesteld door de Europese Referentie Laboratoria (5 µg/L). Dit toont de relevantie van deze grenswaarde aan. Bijkomend kon aan de hand van het metabolietenprofiel in de urinestalen een duidelijk onderscheid gemaakt worden tussen de verschillende stressomstandigheden. In totaal werden 169 differentiërende metabolieten gekarakteriseerd afhankelijk van het soort stress. Dit metabolietprofiel kan ingezet worden om onbekende urinestalen te classificeren naargelang de stress status van het dier, wat leidt tot een betere interpretatie van analyseresultaten van urinestalen met prednisoloneresiduen.

Hoofdstuk V – In dit hoofdstuk werden de toepasbaarheid en geschiktheid nagegaan van de prednisolone/cortisol concentratieverhouding en de 20β-dihydroprednisolone concentratie als

potentiële screeningsmerkers om zo de oorsprong van prednisolone te bevestigen. Hiervoor werd de farmacokinetiek en urinaire excretie van prednisolone, prednisone, 20α-dihydroprednisolone en 20β-dihydroprednisolone na orale en intramusculaire groeibevorderende en therapeutische prednisolone behandeling en farmacologisch geïnduceerde cortisol geëvalueerd in een uitvoerige *in vivo* studie. Daarnaast werd de invloed onderzocht van de verschillende behandelingen op de urinaire concentraties van cortisol en zijn metabolieten. Deze behandelingen leidden tot een significante negatieve feedback voor cortisol en zijn metabolieten. Uit de resultaten bleek de metaboliet 20β-dihydroprednisolone ongeschikt wegens de grote variatie in data en omwille van de afwezigheid van niet significante verschillen tijdens de verschillende behandelingen. De prednisolone/cortisol ratio bleek daarentegen wel een veelbelovende screeningsmerker. Daarnaast bestaat nog steeds de nood aan een voldoende selectieve/specifieke biomerker die het onderscheid tussen endogene vorming en exogene toediening toelaat.

Hoofdstuk VI – In dit hoofdstuk werden vier potentiële biomerkers in urine opgehelderd. Deze zouden het mogelijk maken om de endogene of exogene oorsprong van prednisolone aan te duiden. Hiervoor werd een metabole strategie toegepast in urine van runderen die achtereenvolgens werden onderworpen aan een groeibevorderende en therapeutische prednisolone behandeling. Na evaluatie van de sensitiviteit, specificiteit, urinaire excretiekinetiek en selectiviteit kon één component weerhouden worden als de meest geschikte biomerker. Gebaseerd op de accurate massa, het isotoopprofiel en het MS/MS spectrum werd deze component potentieel geïdentificeerd en zou kunnen dienen als biomerker voor exogene prednisolone toediening.

Hoofdstuk VII – In dit hoofdstuk werden de algemene conclusies en toekomstperspectieven van dit onderzoek geformuleerd. Hierbij werd het nut van het bijnier cel-model en *in vitro* modellen, gebaseerd op de lever, voorgesteld om het onderliggende mechanisme van prednisolonevorming nader te onderzoeken. Alvorens de voorgestelde biomerker (Hoofdstuk VI) kan geïmplementeerd worden als efficiënte screeningstest in nationale controleplannen, dringt zich een grondige validatie op van deze voorgestelde biomerker, in een onafhankelijke dataset.

CURRICULUM VITAE

Curriculum vitae

PERSONALIA

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EDUCATION

Special Research Fund (BOF) (January – September 2015)

- 'Endogenous formation and metabolism of glucocorticoids in cattle'
  Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis

PhD research (2012 - 2014)

- 'Endogenous formation and metabolism of glucocorticoids in cattle'
  Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis

Dehousse-scholarship (October - December 2011)

- 'Endogenous formation and metabolism of glucocorticoids in cattle'
  Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis

Higher education (2006 - 2011)

- 1st, 2nd, 3rd Bachelor in Pharmaceutical Science,
  Ghent University, Faculty of Pharmaceutical Sciences
- 1st and 2nd Master of Science in Pharmaceutical Care
  Ghent University, Faculty of Pharmaceutical Sciences
- Masterthesis (2010): "Voorkomen en endogene vorming van het synthetische thyreostaticum, thiouracil, in diverse biologische matrices". Ghent University, Faculty of Veterinary Medicine

SCIENTIFIC PUBLICATIONS

- **De Clercq, N**.; Vanden Bussche, J.; Van Meulebroek, L.; Croubels, S.; Delahaut, P.; Buyst, D., Martins, J., Stahl-Zeng, J.; Vanhaecke, L. (2015). Metabolic fingerprinting reveals a novel candidate biomarker for prednisolone treatment in cattle. Accepted by Metabolomics.
- **De Clercq, N**.; Van Meulebroek, L.; Vanden Bussche, J.; Croubels, S.; Delahaut, P.; Vanhaecke, L. (2015). The impact of stress on the prevalence of prednisolone in bovine urine: A metabolic fingerprinting approach. Accepted by Journal of Steroid Biochemistry and Molecular Biology, doi:10.1016/j.jsbmb.2015.08.026.
- Van Meulebroek, L.; Vanden Bussche, J.; **De Clercq, N**.; Steppe, K.; Vanhaecke, L. (2015). A Metabolomic Approach to Unravel the Regulating Role of Phytohormones Towards Carotenoid Metabolism in Tomato Fruit. *Metabolomics*, 11(3), 667-683.
- Vanden Bussche, J.; Decloedt, A.; Van Meulebroek, L.; **De Clercq, N**.; Lock, S.; Stahl-Zeng, J.; Vanhaecke, L. (2014). A novel approach to the quantitative detection of anabolic steroids in bovine muscle tissue by means of a hybrid quadrupole time-of-flight-mass spectrometry instrument. *Journal of Chromatography A*, 1360, 229-239.
- **De Clercq, N**.; Vanden Bussche, J.; Croubels, S.; Delahaut, P.; Vanhaecke, L. (2014). Development and validation of a high resolution mass spectrometry based method to study the long-term stability of natural and synthetic glucocorticoids in faeces. *Journal of Chromatography A*, 1336, 76-86.
- **De Clercq, N**.; Vanden Bussche, J.; Croubels, S.; Delahaut, P.; Vanhaecke, L. (2013). A validated analytical method to study the long-term stability of natural and synthetic glucocorticoids in livestock urine using untra-high performance liquid chromatography coupled to orbitrap-high resolution mass spectrometry. *Journal of Chromatography A*, 1301, 111-121.
- Vanhaecke, L.; Van Meulebroek, L.; **De Clercq, N**.; Vanden Bussche, J. (2013). High resolution orbitrap mass spectrometry in comparison with tandem mass spectrometry for confirmation of anabolic steroids in meat. *Analytica Chimica Acta*, 767, 118-127.
- Vanden Bussche, J.;Kiebooms, J.; **De Clercq, N**.; Deceuninck, Y.; Le Bizec, B.; De Brabander, H.; Vanhaecke, L. (2011). Feed or Food Responsible for the Presence of Low-Level Thiouracil in Urine of Livestock and Humans? *Journal of Agricultural and Food Chemistry,* 59(10), 5786-5792.

SCIENTIFIC TRAINING AND ACTIVITIES

International conference:

- 11th Annual International Conference of the Metabolomics Society, June 2015, San Francisco, USA.
  Poster presentation: 'Metabolic fingerprinting to reveal a novel biomarker for prednisolone treatment in cattle'
- 7th International Symposium on Hormone and Veterinary Drug Residue Analysis, June 2014, Ghent, Belgium.
  Oral presentation: 'Metabolomic fingerprinting of glucocorticoids in urine by UHPLC-Orbitrap-MS, Q-Orbitrap-MS and Qq-ToF-MS'
- 13th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC), January 2014, Bruges, Belgium.
  Poster presentation: 'Metabolomic profiling of the glucocorticoid status of Holstein-Friesian cows by U-HPLC-HR-Orbitrap MS upon administration of prednisolone'
- 6th International Symposium on Recent Advances in Food Analysis (RAFA), November 2013, Prague, Czech Republic.
  Oral presentation: 'High-resolution mass spectrometry for metabolomic profiling of the glucocorticoid status of Holstein-Friesian cows after administration of prednisolone'
- Trends in Food Analysis VII Symposium (KVCV), September 2013, Ghent, Belgium.
  Poster presentation: 'Metabolomic profiling of the glucocorticoid status of Holstein-Friesian cows by U-HPLC-HR-Orbitrap MS upon administration of prednisolone'
- 14th International Symposium on Advances in Extraction Technologies (ExTech), September 2012, Messina, Italy.
  Oral presentation: 'Plackett-Burman design: a tool to develop a generic extraction for U-HPLC-Orbitrap-MS analysis of glucocorticoids in livestock urine'
- Conference on Residues of Veterinary Drugs in Food (EuroResidue VII)**,** May 2012, Egmond aan Zee, The Netherlands.
  Poster presentation: 'Endogenous formation of glucocorticoids in livestock urine: a stability study'

National and International training

- Inter-university pharmacokinetic and pharmacodynamic course. Fundamental Principles and Application to Contemporary Drug Development, April 2015, Louvain, Belgium.
- Q Exactive Operations Training Course, ThermoFisher Scientific, December 2013, Ghent, Belgium.
- AB Sciex Course: Basic Triple TOF® 5600 Small Molecule Training, June 2013, Darmstadt, Germany.
- Intensive Programme For Advanced Residue Analysis in Food (IPARAF Course), March - April 2012, Nantes, France.

Doctoral schools of Life Sciences and Medicine

- Project Management - Cluster Career Management, January 2014, Ghent University
- Communication Skills:
  - Basic module, October 2012, Ghent University.
  - Communication skills: Conflict Handling, April 2013, Ghent University.
- Basic statistics in research:
  - Introduction SPSS, October 2012, Instituut voor Permanente vorming (IVPV)
  - Module 1: Basic statistics, November - December 2012 (IVPV)
  - Module 3: Experimental setup, April - June 2013 (IVPV)
- Statistics - Introduction to SPSS, October 2012, Ghent University.
- Clinical studies: study design, implementation and reporting, August 2012, Ghent University.

Tutor of Master students

- 2011 – 2015: One thesis student from the Faculty of Pharmaceutical Science
  Two thesis students from the Faculty of Veterinary Medicine

DANKWOORD

All you need is

faith

trust

and...

a Little Magic 

Zoals jullie weten hou ik het graag kort en bondig en daarom: bedankt iedereen!

In een land vol pillen en grillen zit ik aan de oever van de siroop tot plots een pratend wit konijn uit den Bussche langskomt en vraagt of ik wil helpen met een queeste. Gezien mijn drang naar avontuur moet ik niet lang nadenken en besluit ik haar te volgen. Na een sprong in het diepe kom ik in de wonderlijke wereld van LCA terecht, waar niets is wat het lijkt.

Voor de poorten staat een Wannemacker en moet ik op mijn blote knieën beloven dat alles wat ik zie vertrouwelijk is en ik nooit iets aan iemand zal vertellen (oeps). Na dit plechtige moment en

het afhandelen van de nodige papierwinkel, ontmoet ik de Energieke Koningin. Ze is zo snel als de wind, streeft naar een succesvol bestaan en gaat voor iedereen door een vuur. Ze helpt me waar nodig en geen moeite is haar te veel. Meteen heb ik een goed gevoel over de opdracht die voor mij ligt en begin ik met goeie moed aan de zoektocht naar de ware aard van prednisolone.

Voor mijn queeste loop ik rond in een labyrinth van eilanden, vreemd geurende labo's en vrolijke koffielokalen. Het wit konijn helpt me op weg en stelt mij voor aan Dupje. Ze lijkt op het eerste zicht normaal maar transformeert op vrijdagavond in Michael J. en kijkt elke dag fier naar haar verzameling lege cola-blikjes. Van het eiland naar het koffielokaal is een kleine stap. Ik ben nog niet goed binnen of de Kwislers springen tevoorschijn. Deze meisjes weten echt alles en zijn ook best grappig. Vooral omdat de ene wat trager praat (waardoor het lijkt alsof ze van ergens ver vandaan komt), terwijl de andere het altijd maar over beren heeft (hoewel ik hier nog geen enkele beer heb zien rondlopen). Bizar. Maar geestig!

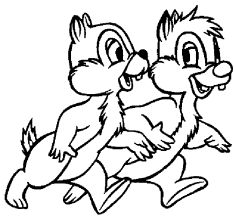
Van queestes oplossen krijgt een mens honger. Dus volg ik mijn neus en kom bij de Chef terecht. Hij is de kok van het eiland en is voornamelijk bekend om zijn 'experimental designs' in de keuken. Vroeger waren ze blijkbaar niet altijd even geslaagd maar hij heeft duidelijk een lange weg afgelegd. Daarnaast blijkt hij ook thuis te zijn in de gokwereld, kan hij sierlijk 5 borden tegelijk dragen zoals een echte butler en is hij meester in het bouwen van hoge stapels. Al snel zijn we verloren in een eindeloze nerdytalk en de slappe lach. Maar het is tijd om verder te gaan en nog meer te ontdekken. Achter de volgende hoek

staan talrijke magische machines die alles kunnen zien, nog scherper dan een glazen bol. Hier worden ze echter 'massaspectrometers' genoemd. Eén knipoog van hun groene lampjes en ik ben meteen verkocht. De elfjes Dirk, Mieke, Beata, Joke, Vicky en Lucie zorgen er dan ook voor dat alles draaiende blijft. Ook weten ze iedereen bij te staan met raad en daad. En ook mij wijzen ze de weg naar mijn volgende bestemming. Bedankt hiervoor.

Om de volgende hypothese te onderzoeken kom ik via een lange gang in een labo terecht. Hier staan een paar sierlijke poezen te miauwen. Het gaat er vrolijk aan toe en ook hun namen klinken als echte magie in de oren: Hemeryck, Kiebooms, Rombouts, Decloedt en Wauters. Ze spinnen en grinnen erop los, met naast hen een sympathieke Chileense vogel. Dan zie ik plots de wijze uil, Johan. Hij neemt mij op zijn schouders en helpt me voorbij de statistische drempel. We vliegen zelfs naar Zele waar we knoeien met steriele potjes en spuiten. Alweer een missie geslaagd!

Ik vervolg het pad zoals het komt, over bergen en dalen, met kronkels en obstakels. Mijn tocht is niet altijd even simpel maar mijn veiligheid is gegarandeerd want er is een blauwhelm waar ik altijd op kan rekenen. En ook al lijkt het heel donker, de vuurvliegjes Anliez, Jojo, Marjo en the boys zorgen voor licht en helpen mij vooruit!

Daarenboven hoef ik mij niet alleen te voelen want ik ben omgeven door zeer goed gezelschap.



Mijn kleinste metgezellen zijn Knabbel en Babbel! Ze zijn hun naam waardig want de een stopt maar niet met babbelen (met alle gevolgen van dien) en de andere proeft alles voor. Ook al zie ik ze niet, ik weet dat ze er altijd voor mij zijn. Ze zijn mijn beste vrienden en zou ze voor geen geld van de wereld kunnen missen! Ook de 'grote zus' en de GVR zorgen voor zeer aangenaam gezelschap op mijn tocht!

Uiteindelijk maakt het niet uit hoever ik vlieg en hoeveel avonturen ik ook meemaak, want naast mij loopt Peter Pan. In zijn vrije tijd is hij tegen wil en dank grafisch ontwerper. De ICT leraar die mij met Word leert werken. De geschiedenisleraar met nuttige maar vooral nutteloze weetjes. De huisman die zorgt voor eten op tafel, een proper huisje en voorziet in een hoog nussyness gehalte. Door vreemden wordt hij vaak mijn broer genoemd omdat we na een tijd op elkaar zijn gaan lijken. Maar voor mij is hij vooral mijn keppe.

Na vier jaar lijkt de missie geslaagd en misschien vind je jezelf niet meteen terug in dit verhaal maar weet dat elk van jullie tot een stukje van de puzzel heeft bijgedragen. Dit cliché klopt nu eenmaal wel. Bedankt.

Nathalie

** Elke gelijkenis met bestaande gebeurtenissen en/of personen berust louter op toeval.*